UNIVERSITAT DE BARCELONA

Identification of suppressors of the constitutive photomorphogenic phenotype of *pifq*: from chloroplasts to rhythmic growth

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Identification of suppressors of the constitutive photomorphogenic phenotype of *pifq*: from chloroplasts to rhythmic growth

Memoria presentada por Guiomar Martín Matas para optar al grado de doctora por la Universidad de Barcelona bajo la supervisión de la Dra. Elena Monte Collado y el Dr. Pablo Leivar Rico. La tesis doctoral se ha realizado en el Centre de Recerca en Agrigenòmica, dentro del programa de doctorado de Genética de la Facultad de Biología, bajo la tutela del Dr. Marc Valls Matheu.

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A mis abuelos

RESUMEN

RESUMEN

Las plantas, durante todo su ciclo vital, adaptan constantemente su desarrollo y crecimiento a los cambios que se producen en el ambiente que las rodea. En periodos de oscuridad, ya sea cuando las plantas germinan bajo la superficie de la tierra o cuando crecen en condiciones diurnas (noche y día), se acumulan en el núcleo los factores de transcripción llamados Phytochrome Interacting Factors (PIFs). Estos factores de transcripción regulan la expresión de miles de genes con el fin de inducir crecimiento y reprimir el desarrollo fotomorfogénico. Tras la germinación en oscuridad, una vez las plantas alcanzan la superficie y se exponen a la luz, los fitocromos, fotoreceptores que perciben la presencia de luz roja y roja lejana, se activan y se traslocan al núcleo, donde inducen la degradación de los factores de transcripción PIFs. La degradación de estas proteínas produce amplios cambios en el transcriptoma de las plantas induciendo el desarrollo fotomorfogénico, caracterizado por la inhibición del crecimiento del hipocotilo, la apertura y expansión de los cotiledones, la apertura del gancho apical y la formación de los cloroplastos. Por ello, plantas mutantes que carecen de cuatro factores PIF (PIF1, 3, 4 y 5) (*pifq*) presentan en oscuridad un fenotipo fotomorfogénico constitutivo y un transcriptoma parecido a plantas crecidas en luz. Al inicio de la tesis, las redes transcripcionales reguladas por los PIFs habían sido descritas, sin embargo, los genes que inician la cascada transcripcional que implementan las funciones celulares que permiten a las plantas desarrollarse fotomorfogénicamente eran desconocidos. Nuestro principal objetivo ha sido determinar estos genes, para ello utilizamos la genoteca FOX (Full-Lenght cDNA Over-eXpressing), que contiene una colección de 10.000 cDNAs independientes de Arabidopsis. La estrategia llevada a cabo fue transformar plantas (*pifq*) con esta librería de cDNAs, y realizar un cribado de supresores del fenotipo fotomorfogénico constitutivo.

Muchos de los mutantes supresores que identificamos codificaban para proteínas de cloroplasto. La formación del cloroplasto es un proceso fuertemente reprimido por las proteínas PIFs e inducido cuando las plantas se exponen a luz. Debido al resultado de nuestro cribado, decidimos estudiar si la formación del cloroplasto regulaba el desarrollo fotomorfogénico. Curiosamente, nuestros resultados demostraron que disfunciones en la biogénesis del cloroplasto producen defectos en el desarrollo fotomorgénico de plantas crecidas en luz, y de mutantes *pifq* crecidos en oscuridad. Además, nuestro trabajo permitió esclarecer el mecanismo molecular por el cual el cloroplasto regula la fotomorfogénesis. Es bien conocido que los cloroplastos regulan la

expresión de genes codificados en el núcleo mediante señales retrógradas que se activan cuando existen disfunciones en el normal funcionamiento del cloroplasto. Nuestros análisis han demostrado que estas señales retrógradas reprimen la expresión génica de un gran porcentaje de genes inhibidos por los PIFs en la oscuridad, y por tanto, inducidos por la luz. Así, las señales retrógradas suprimen el desarrollo fotomorfógenico mediante la represión del programa transcripcional inducido por la luz. Entre los genes coregulados por los PIFs/luz y las señales retrógradas, encontramos el factor de transcripción GLK1, el cual juega un papel principal regulando la fotomorfogénesis aguas abajo de ambas señales. Por tanto, mediante la regulación transcripcional de *GLK1*, los PIFs y las señales retrógradas ajustan el desarrollo fotomorfogénico al estado funcional del cloroplasto. Ya que es sabido que el aparato fotosintético se daña cuando recibe demasiada luz, nosotros demostramos que el mecanismo por el cual las señales retrógradas regulan la fotomorfogénesis ayuda a sobrevivir a las plántulas que germinan en ambientes con intensidades de luz muy elevadas, con el fin de ralentizar su desarrollo fotomorfogénico reduciendo la superficie de tejido fotosintético que las plántulas exponen para la captación de luz.

Dado que no se conocía el papel de GLK1 como regulador de la fotomorfogénesis, decidimos estudiar la función de GLK1 como inductor de la fotomorfogénesis en respuesta a luz. Nuestros resultados han identificado al factor de transcripción BBX16 como un importante regulador de este proceso que actúa aguas abajo de GLK1. Este factor de transcripción induce solo los aspectos morfológicos que suceden en la planta en respuesta a la luz y en respuesta a señales retrógradas, sin regular la formación del aparato fotosintético.

Al mismo tiempo, ya que uno de los mutantes supresores del fenotipo fotomorfogénico constitutivo codificaba para el factor de transcripción CDF2, decidimos centrarnos en la función de esta subfamilia de factores de transcripción Dof en la regulación de la fotomorfogénesis. Nuestros estudios revelan que uno de sus miembros, CDF5, es un importante represor del desarrollo fotomorfogénico. En condiciones diurnas de día corto, las plantas crecen principalmente al final de la noche. Nuestros resultados han determinado que la expresión de CDF5 se induce al final de la noche, mediante la activación transcripcional directa por parte de PIF1, 3, 4 y 5, para favorecer el crecimiento durante esas horas. En paralelo, PRR5 y PRR7, componentes del reloj circadiano, reprimen su transcripción durante las primeras horas de la noche, y por tanto evitan que las plantas elonguen el hipocotilo durante ese periodo.

En resumen, los resultados de esta tesis nos han permitido identificar nuevos reguladores del desarrollo fotomorfogénico que actúan como intermediarios de la cascada de señalización iniciada

por los factores de transcripción PIF. Estos resultados permiten ampliar el conocimiento de la función transcripcional de los PIFs, mostrando ejemplos específicos de genes tanto inducidos (CDF5) como reprimidos (GLK1). Además, nuestro trabajo ha permitido determinar la función del cloroplasto como regulador del desarrollo fotomorfogénico, estableciendo la conexión molecular y funcional entre la señalización lumínica y las señales retrógradas, lo cual establece además las bases para posteriores estudios que permitan profundizar en la función de los PIFs y de las proteínas que median las señales retrógradas.

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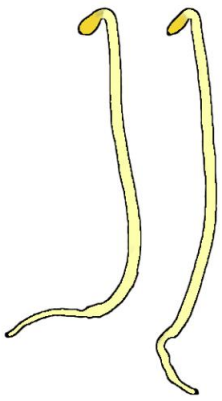


INTRODUCTION

Plants are extremely dependent on sunlight: On one hand, plants are photoautotrophic organisms that harvest energy from sunlight and use it to generate chemical energy; this energy is then used by plants to drive growth, development and reproduction. On the other hand, plants are sessile organisms that are exposed to a wide range of fluctuating environmental conditions. As plants cannot run away from these variations, they must constantly adapt their development to the surrounding environment. Plants perceive seasonal and local environmental information from sunlight. Therefore, they have evolved multiple sensor systems and signaling pathways that confer them the ability to interpret and transduce the light information to changes in nuclear gene expression, which in turn produce readjustments in plant development.

1 LIGHT AS A SOURCE OF INFORMATION

Plants are able to monitor different aspects of the light emitted by the sun, including its quality (spectral composition), quantity, duration (depending on the season and latitude) and direction. The perception of these aspects permits plants to obtain information about the surrounding environment. Plants perceive this informational light by a variety of photoreceptors systems, each of them activate unique and overlapping signaling pathways that confer the ability to adjust developmental and many physiological responses, such as: induction of germination, seedling deetiolation, phototropism, vegetative development, shade avoidance and floral transition (Jiao *et al.*, 2007; Kami *et al.*, 2010).

Molecular responses that occur after light exposure are very rapid (within minutes) and extensive: approximately 20% of the genes in the Arabidopsis genome change their expression in response to light (Jiao *et al.*, 2005; Ma *et al.*, 2005). This high responsiveness to light exposure confers plants the ability to adapt their development to the surrounding environment rapidly and extensively.

1.1 Plant Photoreceptors

Photoreceptors are chromoproteins composed of an apoprotein bound to a variety of chromophores, with the exception of UVR8 class (see below) in which two tryptophan residues act as chromophore (O'Hara and Jenkins, 2012). The chromophore determines the characteristic

absorption spectra in which each photoreceptor becomes active. When photoreceptors perceive light, they change their conformation, conferring them different molecular properties that permit to initiate photoreceptor-mediated signaling pathways (Möglich *et al.*, 2010; Li *et al.*, 2012).

Three classes of photoreceptors have been identified in Arabidopsis based on their absorption spectra: (i) phytochromes (phys), primarily responsible for absorbing the red (R) and far-red (FR) wavelengths (600-750 nm) (Quail, 2010), (ii) three subtypes of photoreceptors perceiving the blue (B)/ultraviolet-A (UV-A) region of the spectrum (320-500 nm): cryptochromes (Liu *et al.*, 2011), phototropins (Fankhauser and Christie, 2015), and three recognized LOV/F-box/Kelch-repeat proteins ZEITLUPE (ZTL), FLAVIN-BINDING KELCH REPEAT F-BOX (FKF), and LOV KELCH REPEAT PROTEIN 2 (LKP2) (Ito *et al.*, 2012); and (iii) UV RESISTANCE LOCUS 8 (UVR8), which was recently shown to be a UV-B (282-320nm) photoreceptor (Rizzini *et al.*, 2011) (Figure 1). Due to their importance in the regulation of deetiolation, in our laboratory we are focused on the phytochrome type of photoreceptors.

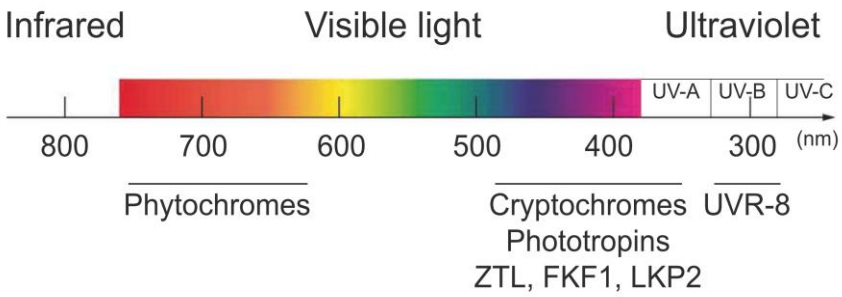


Figure 1. Diagram of light visible spectrum. The absorption spectra (nanometres) of each class of photoreceptors are shown on the bottom part of the diagram.

1.1.1 Phytochromes

1.1.1.1 Structure and dynamics of phytochromes.

Arabidopsis possesses five phytochromes (PHYA-PHYE) that exist as homodimers (Sharrock and Quail, 1989). Each monomer is composed of an apoprotein whose amino terminus is covalently bound to a phytochromobilin chromopher, whereas the carboxi terminus is involved in dimerization processes (Nagatani, 2010).

Phytochromes have two reversible conformations: Pr form, which absorbs red light and is biologically inactive, and the form Pfr, which is active and perceive far red light(Rockwell *et al.*, 2006) (Figure 2).

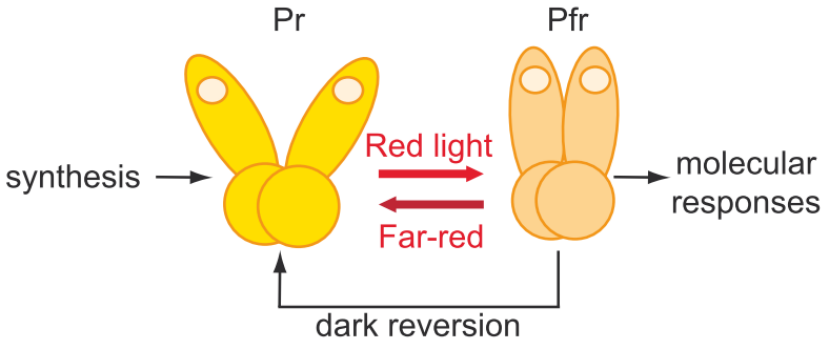


Figure 2. Scheme of phytochrome photoconversion. In dark yellow the Pr inactive form, able to absorb red light. After light absorption, Pr is photoconverted to the Pfr biological active form, represented in light orange. Pale circles represent the associated chromophore to each polypeptide.

Phytochromes are synthesized in the inactive form, which localizes in the cytosol. After absorbing red light, they change their conformation to the Pfr active form, which translocates to the nucleus (Kircher *et al.*, 1999; Yamaguchi *et al.*, 1999; Kircher *et al.*, 2002). Plants grown in continuous light establish a photoequilibrium between the Pfr and Pr forms that depends on the ratio of Red:Far-red light (Figure 2). In this way, plants are able to distinguish the quality of the red light– which changes depending on the time of the day or under vegetation shade environments– and signal this information to the nucleus. Moreover, when plants are exposed to prolonged periods of darkness, the Pfr form is slowly converted to the Pr form, in a process known as dark reversion (Figure 2) (Li *et al.*, 2011).

1.1.1.2 Phytochrome signaling pathways

Once in the nucleus, photoactivated phytochromes initiate a wide range of molecular mechanism that trigger changes in nuclear gene expression through the interaction with other proteins (Bae and Choi, 2008). Among these molecular mechanisms there are two key regulatory pathways:

- **COP1**: light-activated phyA and phyB disrupt COP1 activity (Sheerin *et al.*, 2015). COP1 is an E3 ubiquitin ligase that represses light signaling in the dark by targeting photoreceptors and downstream transcription factors for ubiquitylation and degradation (Li and Deng, 2005). One of the transcription factors targeted for degradation is HY5, an important regulator that induce the expression of light-regulated genes (Oyama *et al.*, 1997; Lee *et al.*, 2007) (Figure 3).

- **Binding to Phytochrome Interacting Factors (PIFs)**: A key regulatory mechanism mediated by phytochromes in the nucleus is the direct interaction with a subgroup of transcription factors named PIFs (Phytochrome Interacting Factors), which accumulate in the dark and interact with the active Pfr phytochrome in the light. This light-induced interaction between the Pfr phytochromes and PIF initiates a cascade of transcriptional changes that allows plants to adjust to the new light environment (Castillon *et al.*, 2007; Leivar and Monte, 2014). For a subset of these PIFs (PIF1, PIF3, PIF4 and PIF5) it has been shown that this interaction triggers their rapid phosphorylation and degradation (Park *et al.*, 2004; Shen *et al.*, 2005; Al-Sady *et al.*, 2006; Oh *et al.*, 2006; Nozue *et al.*, 2007; Lorrain *et al.*, 2008; Shen *et al.*, 2008; Ni *et al.*, 2014). Because of the relevance of PIF transcription factors regulating light-induced development, we discussed below their molecular and biological functions.

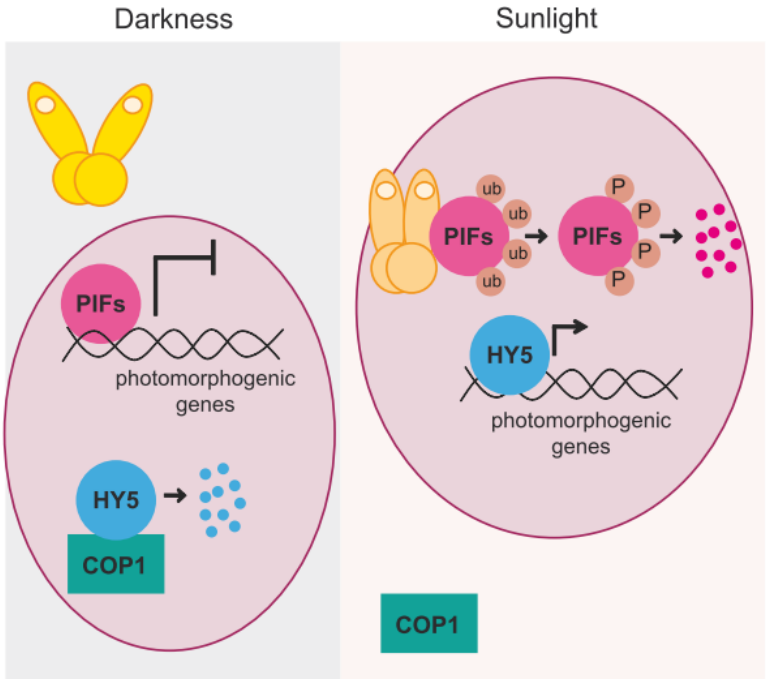


Figure 3. Simplified scheme of phytochrome signalling pathways that involves PIFs and COP1/HY5 proteins. In the dark, inactive form of phytochromes is located in the cytosol and PIFs are active in the nucleus repressing transcription of light-induced genes. Moreover, COP1 is active degrading positive regulators of photomorphogenesis. By contrast, in the light phytochromes are active and translocate to the nucleus where degrade PIF proteins, in addition, expression of light-induced genes is activated by positive transcription factors such as HY5.

1.2 Phytochrome Interacting Factors (PIFs)

Phytochrome interacting factors are transcription factors that are constitutively located in the nucleus and belong to a fifteen-member subfamily of the Arabidopsis bHLH (basic-helix-loop-

helix) superfamily (Toledo-Ortiz *et al.*, 2003), seven of which are PIFs (PIF1, PIF3, PIF4, PIF5, PIF6, PIF7 and PIF8). PIF members are characterized by their ability to interact with the active form of phytochrome B; in addition, PIF1 and PIF3 have a Phytochrome A binding site (APA), which confer them the ability to bind to phytochrome A (Leivar and Quail, 2011).

PIFs, as members of the bHLH family of transcription factors, have two characteristic regions: a basic domain that binds to DNA, and an acid region (HLH), related with the formation of homodimers and heterodimers with other members of the bHLH subfamily. Through their basic domain, these transcriptions factors bind to the G-box DNA regulatory elements (CACGTG) and the E-box variant (CACATG and CATGTG) that has been called PBE-box (for PIF-binding E-box). These regulatory elements are present in the promoters of many light-regulated genes (Martínez-Garcia *et al.*, 2000; Hornitschek *et al.*, 2012; Zhang *et al.*, 2013; Pfeiffer *et al.*, 2014).

1.2.1 Role of PIFs regulating gene expression

Given that PIFs are able to directly bind both DNA and photoactivated phytochromes, they are crucial to bridge light and nuclear gene expression (Leivar and Monte, 2014). Therefore, because of their relevance mediating light signaling, many transcriptomic studies have been done comparing gene expression of WT plants with single or multiple mutants of PIF proteins (Monte *et al.*, 2004; Moon *et al.*, 2008; Leivar *et al.*, 2009; Lorrain *et al.*, 2009; Shin *et al.*, 2009; Nozue *et al.*, 2011; Leivar *et al.*, 2012; Oh *et al.*, 2012; Zhang *et al.*, 2013; Pfeiffer *et al.*, 2014). Gene expression comparison of dark-grown *pifq* and WT seedlings showed that 80% of the misregulated genes in *pifq* were also misregulated in the same direction in light- compared to dark-grown WT seedlings. Therefore, light-induced genes, are repressed by PIFs in the dark, whereas in *pifq* dark- and WT light-grown seedlings these genes are expressed (Figure 4, top pannel). In contrast, light-repressed genes, are induced by PIFs in the dark, and downregulated in *pifq* dark- and WT light-grown seedlings (Figure 4, bottom pannel). This result indicated that PIFs are important regulators of light-induced transcriptional network in the dark (Leivar *et al.*, 2009). Among the subset of light and PIF regulated genes, there are many genes related to chloroplast biogenesis, hormone signaling pathways, growth, and metabolic genes that are involved in the transition from the heterotrophic to photoautotrophic development. These genes are responsible to induce the morphological and physiological adaptations to light (photomorphogenesis).

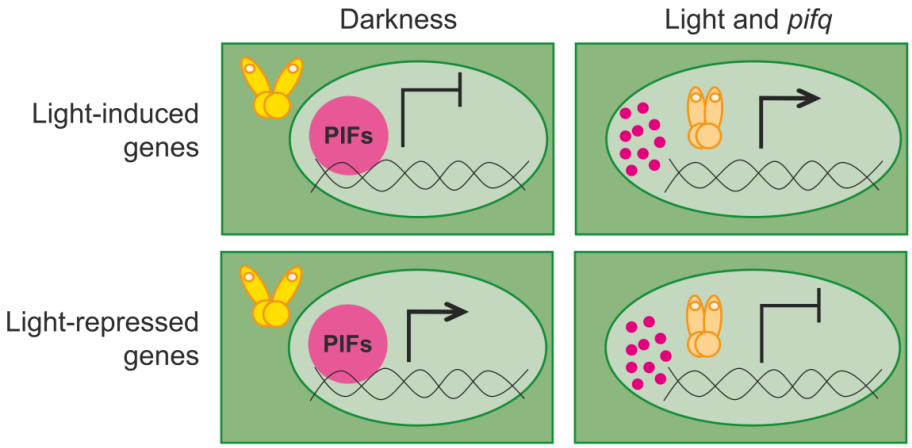


Figure 4: PIF function regulating nuclear gene expression. PIFs repress in the dark (left) light-induced genes (up) and induce expression of light-repressed genes (bottom). In the light or *pifq* mutants (right), the absence of PIFs proteins produce the reversion of the dark transcription pattern.

2. LIGHT AS A SOURCE OF ENERGY (PHOTOSYNTHESIS)

Light energy is captured by complexes composed by chlorophyll and carotenoid molecules bound to proteins. Each of these complexes constitutes a photosystem, which are embedded in the thylakoid membranes of chloroplasts. Light harvesting complexes inside the photosystems harvest sunlight and transfer excitation energy to the core complex, where light energy is transduced into chemical energy (Figure 5).

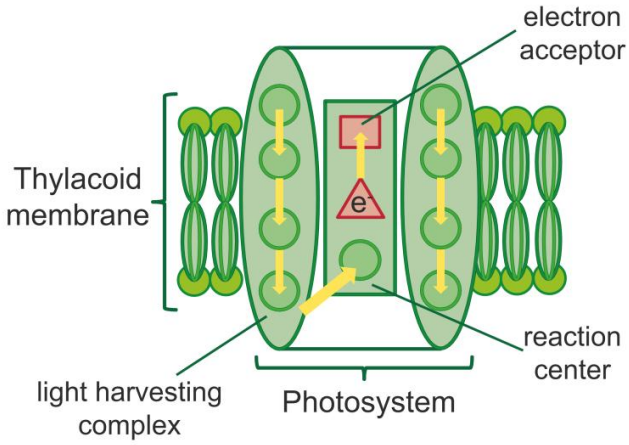


Figure 5: Representative scheme of the photosystem structure. Photosystems are located in thylakoid membranes and are formed by light harvesting complexes. Inside these complexes light is perceived by pigment molecules (indicated as green circles), and transferred to the reaction center, where photons are transferred to electrons.

Electrons captured in the core complex are transferred to different kinds of protein complexes situated in the thylakoid membrane; this electron flow creates a proton gradient across the

chloroplast membrane, which is used to generate ATP by the ATP synthase. In addition, the electron flow is used in an ultimate reaction to reduce NADP to NADPH. Then, the ATP and NADPH generated are used to carry out the Calvin cycle, generating sucrose (Aro and Andersson, 2001).

2.1 Acclimation processes

Photosynthesis is a complex process that can be affected by changes in: light intensity, which modulates capacity of photosystems to harvest light; temperature, because affect the efficiency of enzymatic reactions performed in the Calvin cycle; and water availability, since water molecules are necessary in the first steps of photosynthesis. Changes in these parameters can override the capacity of photosynthesis to process the harvested light, affecting photosynthesis efficiency and producing overexcitation in the photosynthetic apparatus, which subsequently can lead to photodamage and oxidative stress. Therefore, plant cells have evolved a number of photoprotective mechanisms that dissipate energy and repair photodamage: (1) pathways that allow for the dissipation of excess energy; (2) systems that detoxify the harmful by-products of excessive light; and (3) mechanisms that reduce the amount of light absorbed by the plant (Pfannschmidt and Yang, 2012; Gordon *et al.*, 2013; Dietz, 2015).

3. RETROGRADE SIGNALING

Given that chloroplast proteins are encoded both in nuclear and chloroplast genome, chloroplasts have evolved a molecular pathway by which they are able to regulate nuclear gene expression of Photosynthetic Associated Nuclear Genes (PhANGs) (Figure 6). This molecular mechanism is known as retrograde signaling (RS) (Woodson and Chory, 2008), and it functions as an acclimation response because it allows chloroplasts to adapt nuclear gene expression of photosynthesis related genes, regulating thus photosynthesis, to his internal status and/or environmental conditions (Pfannschmidt and Yang, 2012).

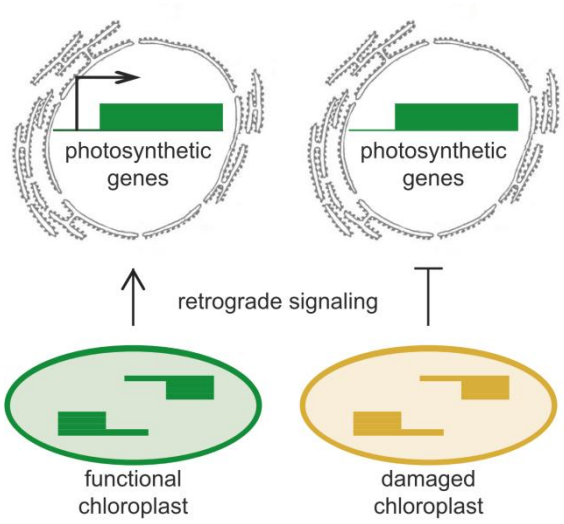


Figure 6: Model for the regulation of PhANG expression by plastids signals. Photosynthetic Associated Nuclear Genes (PhANG) expression is activated in seedlings containing well-functioning chloroplasts (left). In seedlings containing dysfunctional chloroplast, plastid signals that inhibit PhANG expression are active, producing a strongly suppression of PhANG expression.

During chloroplast biogenesis, a light-induced process, the expression of chloroplast and nuclear genomes has to be correctly coordinated to ensure correct stoichiometry of the chloroplast proteins that forms complexes in the chloroplast. At this moment, retrograde signaling informs the nucleus about chloroplast developmental status to correctly adjust nuclear expression of chloroplast proteins (*biogenic control).* In addition to this transcriptomic regulation, retrograde signals have been suggested to affect other aspects of development that are also light-regulated, such as the leaf morphology or the cotyledon expansion and aperture in early seedling development (Ruckle and Larkin, 2009; Cottage *et al.*, 2010). However, little is known about the extent, regulatory mechanisms of plastid and nucleus signal integration, and biological relevance of these signals (Larkin, 2014).

Moreover, external stimulus such as excess light or drought stress (Estavillo *et al.*, 2011) can alter the internal status of functional chloroplast. When damaged, H_2O_2 (Laloi *et al.*, 2006), singlet oxygen (Wagner *et al.*, 2004), or the plastid redox state (Pfannschmidt *et al.*, 2003) can act as retrograde signals regulating expression of genes that encode for proteins that form the photosynthetic apparatus to readjust the photosynthetic capacity of plants. In addition, recently it has been shown that these retrograde signals also regulate expression of genes that belong to metabolic pathways or stress responses (Bräutigam *et al.*, 2009; Xiao *et al.*, 2013). This regulation by retrograde signaling of nuclear gene expression in response to external stimuli that damage the chloroplast is known as *operational control* (Pogson *et al.*, 2008).

4. PHOTOMORPHOGENESIS

Photomorphogenesis, which comprises many aspects of plant growth and development, allows plants to adapt their growth pattern to light environments, for example, ensuring optimal exposure of photosynthetic plant parts to light and allowing maximal rates of carbon fixation.

4.1 First exposure to light: seedling deetiolation

Plants germinated in the dark under the soil surface, undergo skotomorphogenic development: seedlings exhibit fast-growing hypocotyls to rapidly reach the soil surface, together with an apical hook, which protect the apical meristem from damage during growth through the soil, and appressed cotyledons with undeveloped chloroplasts. Plants emerging from the soil surface perceive light for the first time. Then, seedlings switch from skotomorphogenic to photomorphogenic development, a process that is known as seedling deetiolation (Arsovski *et al.*, 2012). At this moment, seedlings rapidly form the photosynthetic apparatus to start to produce energy and avoid photodamage. In addition, seedlings adapt their morphology to enhance light capture for photosynthesis, inhibiting hypocotyl elongation and stimulating cotyledon separation and expansion. The differences between skotomorphogenesis and photomorphogenesis result from changes in the expression of specific genes (Jiao *et al.*, 2005; Ma *et al.*, 2005). As explained before, PIF proteins are master regulators of the transcriptomic changes that occur during deetiolation, being crucial repressors of photomorphogenesis in the dark (Figure 4). Consequently, a mutant lacking PIF1, PIF3, PIF4 and PIF5 (*pifq*) displays a constitutive photomorphogenic phenotype in the dark, characterized by opened hook, expanded and opened cotyledons, shorter hypocotyls and developed chloroplast (Leivar *et al.*, 2008; Shin *et al.*, 2009) (Figure 7).

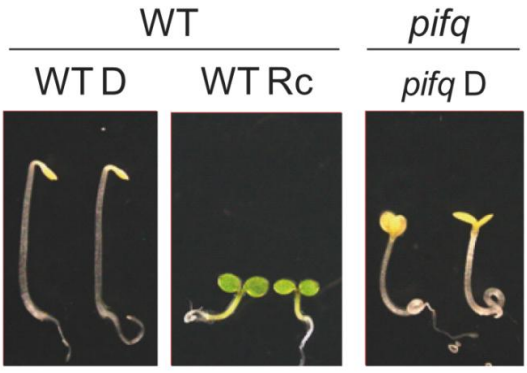


Figure 7: PIFs are required to promote growth in etiolated seedlings. Wild-type seedlings (WT) grown for 2 days in the dark (WT D) (left) or in continuous red light (WT Rc) (middle). On the right are shown *pifq* seedlings grown for 2 days in the dark (*pifq* D).

4.1.1 B-BOX, regulators of seedling deetiolation

Many transcription factors have been proposed as regulators of seedling deetiolation in the light, some of them belong to the family of B-box zinc finger of transcription factors (Bowler *et al.*, 2013). This family of transcription factors is formed by 32 members, classified in five different structural groups (Khanna *et al.*, 2009; Crocco and Botto, 2013) (Figure8). Structural differences depend on the presence of one or two B-box motifs in the B-box domain, which are predicted to be involved in protein-protein interactions and transcriptional regulation. In addition, these differences relay in the presence or absence of the CTT and valine-proline (VP) domains. The first one is present in 17 of the 32 members, and has important functions in transcriptional regulation and nuclear protein transport. The VP motif allows the interaction with COP1.

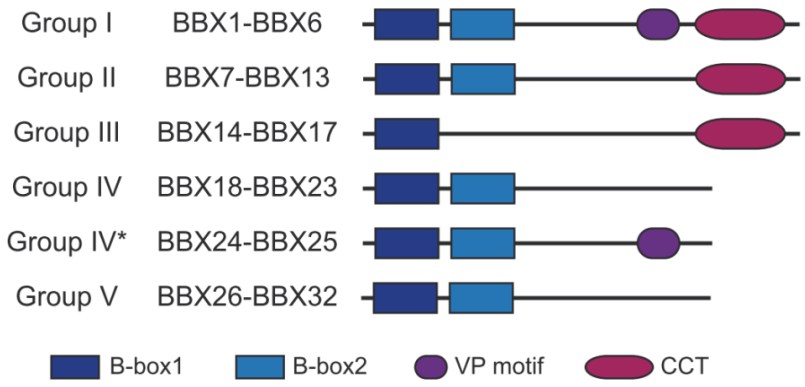


Figure 8: Structure Classification of the B-Box Family. Subfamily of 32 Arabidopsis BBX proteins arranged by structural group. Members and domain motifs present in each subgroup are shown.

BBX proteins are involved in seedling deetiolation, controlling hypocotyl growth, anthocyanin production, chlorophyll accumulation, lateral root growth, and cotyledon and hook unfolding. Specifically BBX4/COL3, BBX20/BZS1, BBX21/STH2, and BBX22/LZF1 are positive regulators of photomorphogenesis (Datta *et al.*, 2006; Datta *et al.*, 2007; Chang *et al.*, 2008; Datta *et al.*, 2008; Fan *et al.*, 2012; Wang *et al.*, 2015). In contrast, BBX23/MIDA10, BBX24/STO, BBX25/STH, BBX32/EIP6, BBX18/DBB1a and BBX19/DBB1b are negative regulators of light signaling (Khanna *et al.*, 2006; Indorf *et al.*, 2007; Kumagai *et al.*, 2008; Holtan *et al.*, 2011; Sentandreu *et al.*, 2011; Gangappa *et al.*, 2013). In addition, it has been shown that light and circadian clock regulates stability and accumulation of BBX proteins (Kumagai *et al.*, 2008).

4.2 Dark to light transition: diurnal conditions

In young seedlings grown under diurnal (day/night) conditions, the light hours photoactivate the plant photoreceptors that signal to the nucleus to induce photomorphogenesis. During this period, a large fraction of the carbon assimilated during the day is stored transiently in the chloroplast as starch for use during the subsequent night. Moreover, the photoactivated photoreceptors repress the developmental program implemented during the dark hours that promote plant growth, especially in the last night hours (Dowson-Day and Millar, 1999; Farré, 2012). This regulation of plant growth in diurnal conditions is in part due to the phytochrome regulation of PIF levels. During the day, phytochromes trigger the decrease in abundance of these PIFs, whereas during the night, the progressive decline in the Pfr levels due to dark reversion allows reaccumulation of PIF1, PIF3, PIF4 and PIF5. This daily regulation of PIFs produces that seedlings do not grow at the same rate during all the diurnal cycle; instead, they have a peak of elongation at the end of the dark hours, coinciding with the maximum in PIF accumulation (Nozue *et al.*, 2007; Soy *et al.*, 2012).

Moreover, the regulation of hypocotyl grown also depends on the number of dark hours within a diurnal cycle, which confers seasonal regulation of elongation. In short days (8 hours light + 16 hours dark) seedlings grow more than in long days (8 hours light + 16 hours dark) (Niwa *et al.*, 2009). PIFs are also involve in this regulation because their protein and RNA accumulation occurs during the night, consequently, the more dark hours the more PIFs accumulate at the end of the night (Niwa *et al.*, 2009; Nozue *et al.*, 2011; Soy *et al.*, 2012). Accordingly, a young seedling grown in short days is longer than that grown in long days. However, *pifq* seedlings grown in short days are more similar to WT seedlings grown in long days (Figure 9), demonstrating that hypocotyl growth that occurs in the night under short day conditions is PIF-dependent.

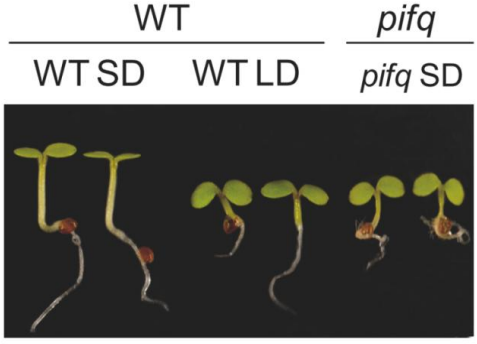


Figure 9: PIFs are required to promote growth under diurnal conditions. Wilde type seedlings (WT) grown for 3 days in short days (SD) (left) or in long days (LD) (middle). On the right are shown *pifq* seedling grown for 3 days in short days.

PIFs induce growth at the end of the night through the transcriptional activation of growth related genes (Nomoto *et al.*, 2012; Soy *et al.*, 2012; Soy *et al.*, 2014). Expression microarray experiments done with *pif4pif5* mutants grown in diurnal conditions (Nozue *et al.*, 2011) permitted to identify the growth regulatory network acting downstream of PIF4 and PIF5. This analysis showed that PIFs induce growth by inducing the expression of genes that belong to the auxin hormone pathway, a plant hormone that contribute to diurnal growth control (Kunihiro *et al.*, 2011; Nozue *et al.*, 2011; Nomoto *et al.*, 2012).

4.2.1 Circadian clock regulation of plant growth

The circadian clock is a biological oscillator that maintains rhythms of about 24 hours. This endogenous rhythm allows plants to predict daily and seasonal environmental changes to coordinate physiological responses to the correct time that they must occur. Thus, circadian clock optimizes internal processes with respect to external conditions (Nagel and Kay, 2012; Shim and Imaizumi, 2015). The biological oscillations are produced by the transcriptional regulation of many proteins that act as central components, this regulation is achieved by complex feedbacks that produce that each component has a window of action of hours (Hsu and Harmer, 2014; Müller *et al.*, 2014). Many biological processes are regulated by circadian clock, including stress responses, hypocotyl growth, carbon fixation or flowering time.

Since many mutants of circadian core proteins have defects in growth (Más *et al.*, 2003; Hemmes *et al.*, 2007; Ito *et al.*, 2007; Niwa *et al.*, 2009), the implications of circadian clock regulating hypocotyl growth have been well studied on the last years. Hypocotyl growth is dependent on the water and carbon availability, which is maximum at the end of the night (Dodd *et al.*, 2005), therefore circadian rhythms favor that hypocotyl elongation occurs at dawn (Nozue *et al.*, 2007). This rhythmic regulation of plant growth is in part produced because circadian core components regulate directly the mRNA levels of PIF4 and PIF5 to peak at dawn (Nusinow *et al.*, 2011; Nakamichi *et al.*, 2012). In addition, circadian clock regulates sucrose levels and gates the response to different hormones (Covington and Harmer, 2007; Arana *et al.*, 2011; Haydon *et al.*, 2013), which have been suggested to regulate PIF protein levels or activity (de Lucas *et al.*, 2008; Stewart *et al.*, 2011).

4.2.2 Cycling DOF factors.

4.2.2.1 DOF family of transcription factors.

DNA binding with One Finger (DOF) proteins is a group of plant-specific transcription factors that contain a 50-amino acid conserved domain in the N-terminal region. This DOF domain corresponds to a C2-C2 zinc finger that binds specifically to the 5'-T/AAAAG-3' sequence motif in the promoters of direct target genes (Yanagisawa and Schmidt, 1999). In contrast, the C-terminal protein region has a highly variable structure, containing protein-protein interaction domains and other regulatory elements.

The DOF family of transcription factors has 32 members (Yanagisawa, 2002; Le Hir and Bellini, 2013), and over the last years some of them have been functionally characterized, elucidating their molecular and biological functions. Interestingly, there is a big diversity in the biological processes that they are regulating, ranging from carbon fixation and nitrogen assimilation (Rueda-López *et al.*, 2008), stomata function and development (Cominelli *et al.*, 2011; Negi *et al.*, 2013), seed germination (Gualberti *et al.*, 2002; Papi *et al.*, 2002), cell cycle (Skirycz *et al.*, 2008), to vascular development(Konishi and Yanagisawa, 2007; Gardiner *et al.*, 2010). Importantly, some of the DOF proteins have been described as positive or negative regulators of photoreceptor signaling (Park *et al.*, 2003; Ward *et al.*, 2005; Khanna *et al.*, 2006; Gabriele *et al.*, 2010).

4.2.2.2 Biological function of Cycling DOF factors.

The cycling DOF Factors (CDFs) is a subgroup of the DOF family of transcriptional regulators that have been described as regulators of flowering time, including CDF1, CDF2, CDF3, CDF4 and CDF5 (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). Among them CDF4 is less similar in their protein products and in their expression profiles (Fornara *et al.*, 2009). Many studies have been done with CDF1, CDF2, CDF3 and CDF5 that had established that these transcription factors are transcriptional repressors of CONSTANS, a gene which is required to promote flowering (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). Interestingly, CDFs are responsible of the induction of flowering that occurs in long days as a consequence of the regulation of their protein levels in a seasonal manner. CDFs proteins are degraded by a complex formed by GIGANTEA (GI) and a F-box protein encoded by FLAVIN BINDING KELCH REPEAT F-BOX PROTEIN 1 (FKF1) (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). FKF1 homologues, including ZEITLUPE (ZTL) and LOV KELCH PROTEIN 2 (LKP2), act redundantly with FKF1 to reduce the amount of CDFs (Fornara *et al.*, 2009). Importantly, this degradation complex is only able to form in the evening under long day

conditions, because in these conditions peak of expression of both proteins coincide, and light activates FKF1. CDF degradation releases repression of CO transcription (Figure 10) (Andres and Coupland, 2012; Song *et al.*, 2013).

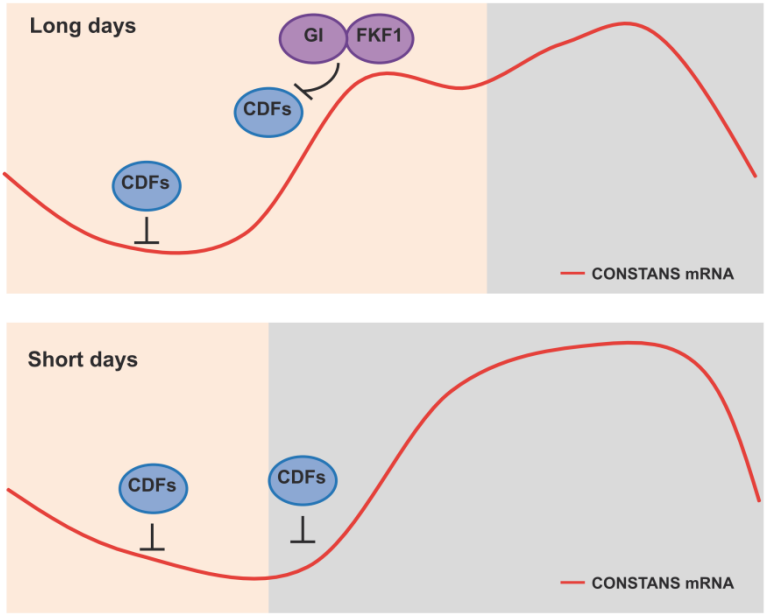
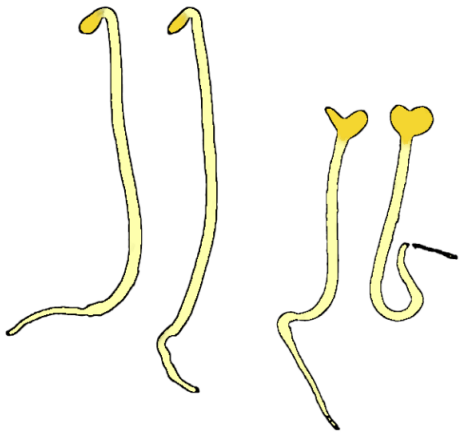


Figure 10: CDF protein levels are regulated in a seasonal manner. In the evening under long day conditions (top) CDF protein are degraded by the complex formed by GIGANTEA (GI) and FLAVIN BINDING KELCH REPEAT F-BOX PROTEIN 1 (FKF1). Due to this reduction of CDF protein levels, *CONSTANTS* expression is activated. In contrast, in short days (bottom) the degradation complex is not formed and CDFs remain active during the evening, repressing the transcription of *CONSTANS*.

Moreover, a recent transcriptome profiling of a mutant lacking CDF1, CDF2, CDF3 and CDF5 (*cdfq*) indicated that genes related to growth and freezing tolerance are misregulated in this mutant, indicating that CDFs contribute to several processes in addition to flowering (Fornara *et al.*, 2015). However, biological relevance and molecular mechanism underlying this transcriptional regulation remain unknown.

OBJECTIVES



OBJECTIVES

At the beginning of this thesis, the PIF-regulated transcriptional networks that operate during photomorphogenesis had been described. However, the primary target genes that implement downstream cellular functions or amplify the PIF signaling pathway had not yet been identified. Therefore the main goal of this work is to identify the downstream effector genes of the PIF transcription factors to elucidate the PIF-regulated transcriptional network that represses photomorphogenic development.

The specific objectives of this thesis were:

1. To identify regulators of photomorphogenesis acting downstream of the PIF transcription factors.

    1.1 To set up and perform a suppressor screen of the constitutive photomorphogenic phenotype of dark-grown *pifq* mutant seedlings.

    1.2 To sequence the genes identified in our genetic screen and study their putative function in the PIF transcriptional network.

    1.3 To functionally characterize selected putative regulators of photomorphogenesis identified in our screen.

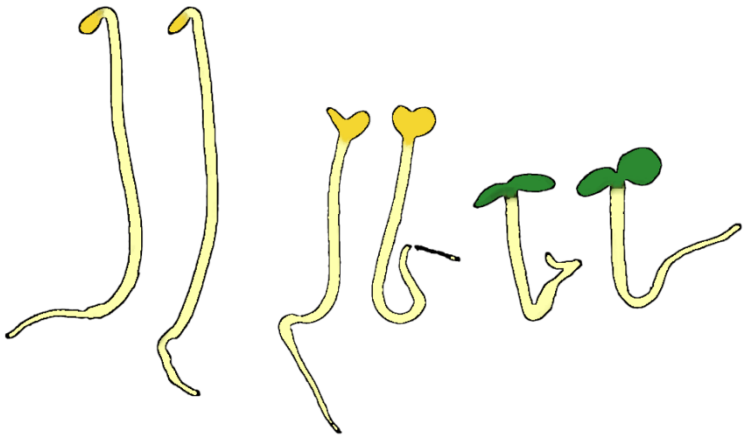
        1.3.1 To study their role in the regulation of photomorphogenesis.

        1.3.2 To study their regulatory interplay with the PIF transcription factors.

In addition, given the function of the genes identified in the suppressor screen, we included an additional objective:

2. To study the interplay of chloroplast biogenesis (a PIF-regulated process) and chloroplast retrograde signaling in the regulation of seedling deetiolation.

RESULTS



RESULTS

1. A suppressor screen to identify new regulators of PIF-mediated seedling deetiolation.

In order to identify new regulators of seedling deetiolation acting downstream of PIF transcription factors, we designed a genetic screen to search for mutants that suppress the constitutive photomorphogenic phenotype of the *pifq* mutant. For this purpose, we first generated a collection of mutagenized *pifq* seedlings, and then we set up a phenotypic screening to identify *pifq* mutants that resemble WT dark-grown seedlings, i.e. with longer hypocotyls and/or closed cotyledons or hooks.

1.1 The FOX hunting system

Since previous studies suggested that at least part of the *pifq* constitutive photomorphogenic phenotype in the dark is due to the downregulation of PIF-regulated genes (Leivar and Quail, 2011), we designed a gain-of-function approach to identify mutants that restore the expression of skotomorphogenic inductors that are downregulated in the *pifq* mutant (Figure 11). This strategy consisted of transformation of *pifq* mutants with a full-length cDNA overexpression (FOX) *Agrobacterium* library (Ichikawa *et al.*, 2006), which contains around 10,000 independent Arabidopsis cDNAs under the control of the strong and constitutive CaMV 35S promoter, and carries a hygromycin resistance gene for plant selection.

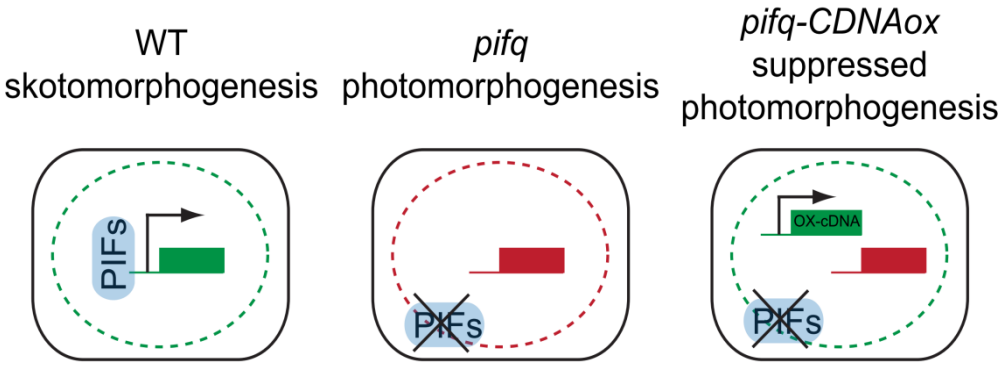


Figure 11. Strategy to search for suppressors of the *pifq* constitutive photomorphogenic phenotype in the dark. In the dark, PIF transcription factors activate the expression of skotomorphogenic inductors (left). In contrast, in *pifq* mutant these inductors are not expressed because of the absence of PIFs (middle), leading to a constitutive photomorphogenic phenotype of *pifq*. Our strategy is to overexpress individual cDNAs under the control of the 35S

promoter in *pifq* mutants (right), with the goal of identifying those that restore their expression levels and therefore the skotomorphogenic phenotype.

Importantly, we reasoned that the FOX hunting system also allows the identification of other classes of mutants such as skotomorphogenic inductors that do not belong to the PIF regulatory network, as the overexpression of these genes might also suppress the *pifq* constitutive photomorphogenic phenotype.

1.2 Generation of a collection of pifq transgenic plants overexpressing approximately 10.000 full-length cDNAs.

The FOX *Agrobacterium* library was used to transform *pifq* plants by the floral dip method, and we obtained transformed T1 seeds to perform the screening. Transformation efficiency was calculated for each round of transformation by growing T1 seedlings on plates containing the transformation selection marker hygromycin, and efficiencies lower than 1.5% were disregarded. To cover twice the size of the FOX library, our initial objective was to screen around 20,000 transformed *pifq* seedlings. Therefore, with a 1.5% transformation efficiency, we needed to screen a population of around 1,333,000 T1 transformed seeds.

1.3 Setting up the screening conditions to identify suppressor-of-pifq mutant phenotypes in darkness.

1.3.1 Balancing time in darkness and seedling survival

pifq mutant seedlings grown for prolonged periods in darkness show a robust photomorphogenic phenotype that facilitates the phenotypic screen. However, prolonged incubation in the dark induces a detrimental photobleaching phenotype upon exposure to light that compromises the seedling survival (Huq *et al.*, 2004; Leivar *et al.*, 2009). Therefore, we needed to balance these two parameters by identifying an incubation time that is long enough to facilitate the phenotypic visualization without inducing a subsequent photobleaching phenotype. Time-course analysis of the seedling survival response indicated that an incubation time of 2 days and 17 hours in darkness fulfilled our screening requirements (Figure 12).

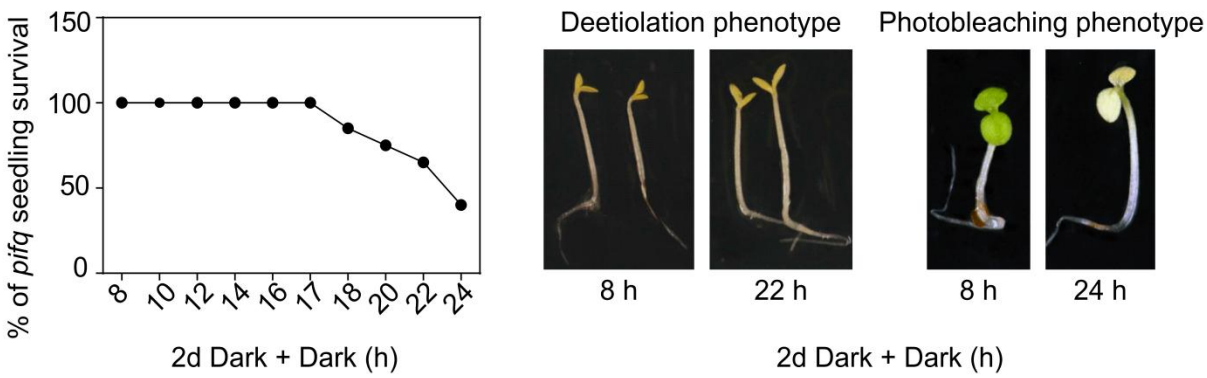


Figure 12. Seedling survival of *pifq* mutants. At least 100 *pifq* seedlings were grown in the dark for the indicated time, and then were transferred to light before determining the seedling survival/photobleaching phenotype. For each incubation time in darkness, we calculated the % of seedling survival by quantifying the number of seedlings that were able to green after 24 hours in the light (left panel). Representative seedlings grown in the dark for 2d+8h or for 2d+22h (middle panel), or seedlings grown in the dark for 2d+8h or for 2d+24h and then transferred to light (right panel).

1.3.2 Use of hygromycin to select for pifq transformants.

In order to eliminate untransformed *pifq* seedlings from the T1 screening population and simplify the phenotypic visualization of the potential suppressors, we decided to use plates containing hygromycin. Phenotypic analysis showed that hygromycin does not significantly affect the constitutive photomorphogenic phenotype of transformed *pifq* seedlings used as controls (Figure 13), and thus we concluded that it can be used in the screening.

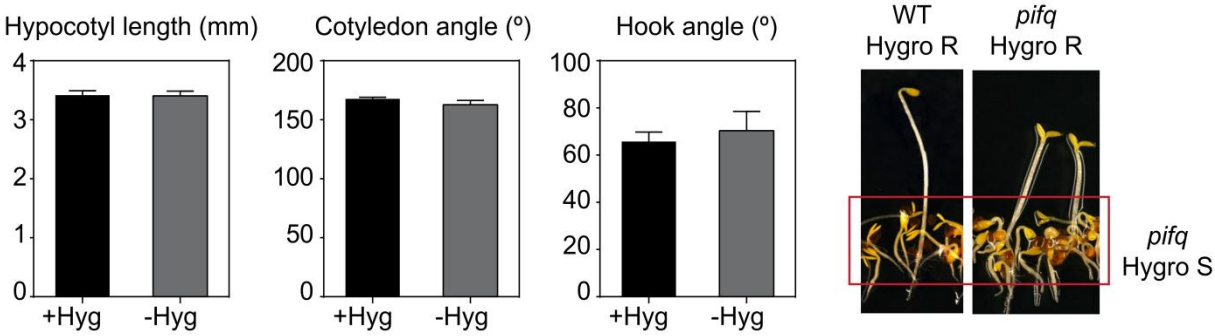


Figure 13. *pifq* phenotypes in the dark are not significantly affected by hygromycin. Phenotypic analysis of control hygromycin resistant *pifq* seedlings grown on plates without (-hyg) or with hygromycin (+hyg) for 2d and 17h in the dark. Data represent mean and standard error from at least 35 seedlings (three left panels). Image represents WT and *pifq* control seedlings resistant to hygromycin (Hygro R) grown in presence of antibiotic (right panel). Hygromycin-sensitive (Hygro S) *pifq* seedlings are not able to develop and are marked with a red box.

1.4 Screening and selection of putative suppressor mutants in the T1 generation.

With the purpose of establishing the range of cotyledon aperture, hook angle, and hypocotyl length considered as suppressor phenotypes in the screening population (T1), we determined the normal distribution of these characters in the *pifq* transformed seedlings. Then we considered putative suppressor phenotypes as those with values that fall out of the 97.5% of the distribution (Figure 14) (i.e. seedlings with hypocotyls longer than 5.61 mm, cotyledon angles less than 11.64 degrees, and/or hook angles below 132.23 degrees).

We used these established criteria to screen a T1 population of 13,260 transformed *pifq* seedlings, which resulted in the selection of 143 putative *suppressors-of-pifq* (*sop*) mutants. The suppressor mutants were longer and/or with closed cotyledons, but none of them had a closed hook.

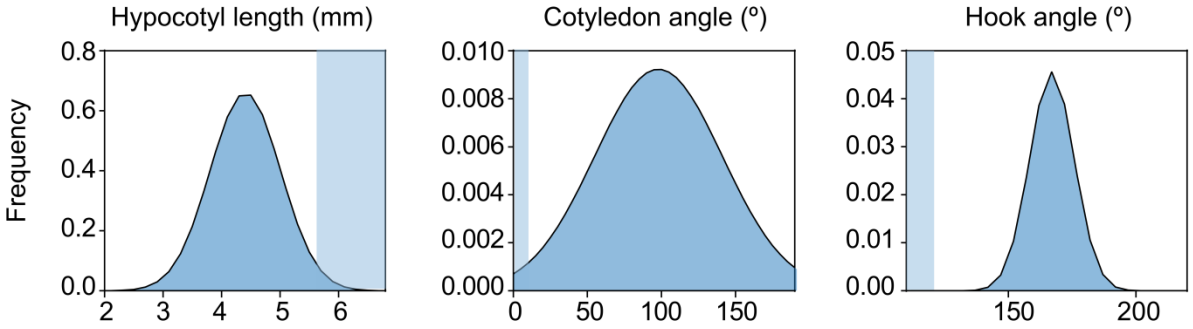


Figure 14. Normal distribution for hypocotyl length (left), cotyledon angle (middle) and hook angle (right) phenotypes of the T1 *pifq* transformed seedlings in the dark. Intervals defined as putative suppressor phenotypes are shown as blue vertical stripes.

In addition, we found 25 seedlings with an enhanced photomorphogenic phenotype, with cotyledon angles extremely open (more than 235 degrees) and/or hypocotyls shorter than 3.35 mm. We decided to select them as putative enhancers of seedling deetiolation and we named them *enhancer-of-pifq (enop)*.

With the intention of avoiding false positives due to an alteration in the germination time, we discarded mutants with longer hypocotyls and more open cotyledons, or with shorter hypocotyls and closer cotyledons, because these phenotypes might correspond, respectively, to *pifq* seedlings that germinated earlier or later than the controls.

1.5 Phenotypic validation of putative regulator mutants in the T2 generation

Next, to confirm the phenotype of the regulator (*sop/enop)* mutants identified in the screening population (T1), we performed a phenotypic study of these mutants in the T2 generation. T2 corresponded to a population in which the presumptive cDNA responsible for the suppressor/enhancer phenotype was segregating, and therefore, the *sop/enop* phenotypes were segregating as shown in Figure 15 for representative validated and not validated *sops*. We only considered validated mutants those with statistically significant different phenotypes compared to the *pifq* control population for hypocotyl length and/or cotyledon angle in two replicate experiments.

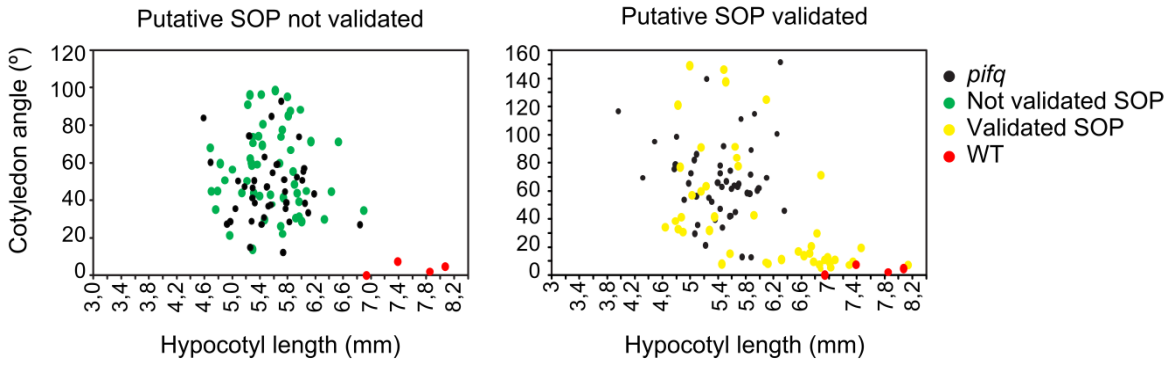


Figure 15. Phenotypic validation of putative regulator mutants in the T_2 generation. A distribution graph of cotyledon angle vs hypocotyl length is shown for a T2 population of representative putative *sop* not validated (left, green circles) and a putative *sop* validated (right, yellow circles). Graph also includes a population of control *pifq* (black circles) and wild type (red circles) genotypes. Validated *sop* seedlings that are more similar to WT presumably correspond to the individuals carrying the cDNA in the T2 segregating population.

As a result of this analysis, 28 putative regulators were validated, corresponding to 20 out of the 143 suppressors (*sop*), and 8 out of the 25 enhancers (*enop*) (Figure 16). Importantly, 40% of these mutants had altered phenotypes both in the hypocotyl and the cotyledons (Figure 16A), suggesting that these mutants are affecting global seedling photomorphogenesis rather than an organ-specific branch.

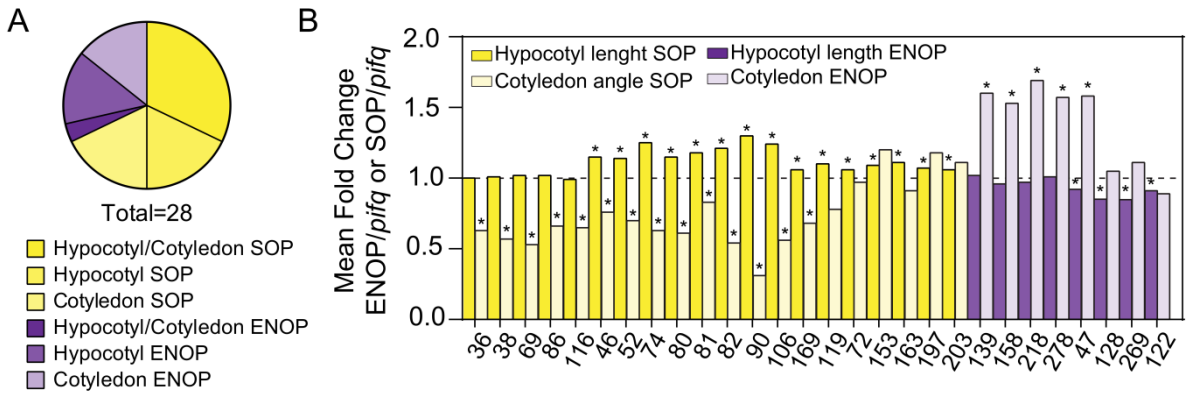


Figure 16. Phenotypic analysis of validated *sop/enop* mutants in the T2 generation. (A) Pie chart showing the percentage of the 28 validated mutants classified by the organ affected (cotyledon and/or hypocotyl) by the suppressor (SOP) or enhancer (ENOP) mutation. **(B)** Hypocotyl length and cotyledon angle phenotypes of the validated suppressors (sops, yellow) and enhancers (enop, purple) represented as a mean fold change to *pifq* (SOP/*pifq* or ENOP/*pifq*). A fold change of 1 indicates no difference compared to *pifq*, and is marked by a dashed line. Means were calculated from at least 50 seedlings. Asterisks indicate statistically differences from *pifq* by Student's *t* test.

1.6 Identification of the candidate SOP/ENOP gene in the validated mutants.

Once we validated the *sop/enop* mutants, we cloned and sequenced the inserted full-length cDNA in each line presumably causing the suppressor/enhancer-of-*pifq* phenotype. First, we used specific primers of the FOX construct to amplify the inserted cDNA from genomic DNA. Then we cloned the PCR product in a TOPO vector, and sequenced the corresponding recombinant clone.

Among the 28 validated mutants, one had two cDNAs inserted in the genome, four of them carried an empty vector of the library without any cDNA, and 23 had a single full-length cDNA. These percentages were similar to those described for the FOX Agrobacterium library (Ichikawa *et al.*, 2006). Table 1 summarizes the AGI number, the annotation and the organ phenotype for each of the 28 identified SOP/ENOPs.

Table 1: List of the validated SOP and ENOP genes. The Arabidopsis Gene Identification number (AGI), the name assigned in the screening (screening name), the annotated description at TAIR (http://www.Arabidopsis.org) website (annotation), and the organ affected by the suppressor or enhancer mutant phenotype (organ phenotype) is shown for each of the validated SOP and ENOP genes.

AGI	Screening name	Annotation	Organ Phenotype
AT1G53180	ENOP 139	Unknown protein	Cotyledon
AT5G50870	ENOP 158	UBC27, UBIQUITIN-CONJUGATING ENZYME 27	Cotyledon
	ENOP 218	Empty vector	Cotyledon
AT5G11730	ENOP 278	N-acetylglucosaminyltransferase family protein	Cotyledon
AT3G13224	ENOP 47	RNA-binding (RRM/RBD/RNP motifs) family protein	Cotyledon / Hypocotyl
AT3G22840	ENOP 128	ELIP1	Hypocotyl
AT1G73220	ENOP 269	ATOCT1, ORGANIC CATION/CARNITINE TRANSPORTER1	Hypocotyl
AT3G58760	ENOP 122	Integrin-linked protein kinase family	Hypocotyl
AT2G36000	SOP 119	EMBRYO DEFECTIVE 3114	Hypocotyl
AT4G03200	SOP 36	Catalytics	Cotyledon
AT2G21280	SOP 38	GIANT CHLOROPLAST 1	Cotyledon
AT3G16250	SOP 69	NDF4, NDH-DEPENDENT CYCLIC ELECTRON FLOW 1	Cotyledon
AT1G60940	SOP 86	SNRK2-10, SNF1-RELATED KINASE 2B	Cotyledon
AT1G29470	SOP 116	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	Cotyledon
AT4G17730	SOP 46.1	ATSYP23, SYNTAXIN OF PLANTS 23	Cotyledon / Hypocotyl
AT1G09690	SOP 46.2	SH3-like family protein	Cotyledon / Hypocotyl
AT2G47760	SOP 52	ALG3, ASPARAGINE-LINKED GLYCOSYLATION 3	Cotyledon / Hypocotyl
AT3G08740	SOP 74	Elongation factor P (EF-P) family protein	Cotyledon / Hypocotyl
AT1G18700	SOP 80	DNAJ heat shock N-terminal domain-containing protein	Cotyledon / Hypocotyl
AT3G13180	SOP 81	NOL1/NOP2	Cotyledon / Hypocotyl
	SOP 82	Empty vector	Cotyledon / Hypocotyl
AT5G39660	SOP 90	CDF2, CYCLING DOF FACTOR 2	Cotyledon / Hypocotyl
AT5G59690	SOP 106	Histone superfamily protein	Cotyledon / Hypocotyl
AT4G32480	SOP 169	DUF506, Protein of unknown function	Cotyledon / Hypocotyl
AT5G10100	SOP 72	TPPI, TREHALOSE-6-PHOSPHATE PHOSPHATASE I	Hypocotyl
AT5G23010	SOP 153	MAM1, METHYLTHIOALKYLMALATE SYNTHASE 1	Hypocotyl
	SOP 163	Empty vector	Hypocotyl
AT4G22690	SOP 197	CYTOCHROME P450, FAMILY 706, SUBFAMILY A, POLYPEPTIDE 1	Hypocotyl
	SOP 203	Empty vector	Hypocotyl

1.7 Functional characterization of validated regulators.

To start to gain insight into the function of validated regulators (SOPs and ENOPs), we analyzed their subcellular localization and biological function based on Gene Ontology annotations available at TAIR (http://www.Arabidopsis.org). Interestingly, although there is a good representation of nuclear/transcription and signaling genes, consistent with possible regulatory functions, unexpectedly the most abundant category corresponded to chloroplast proteins (Figure 17). This enrichment suggested that chloroplast-localized proteins are able to regulate photomorphogenic development in the *pifq* mutant background.

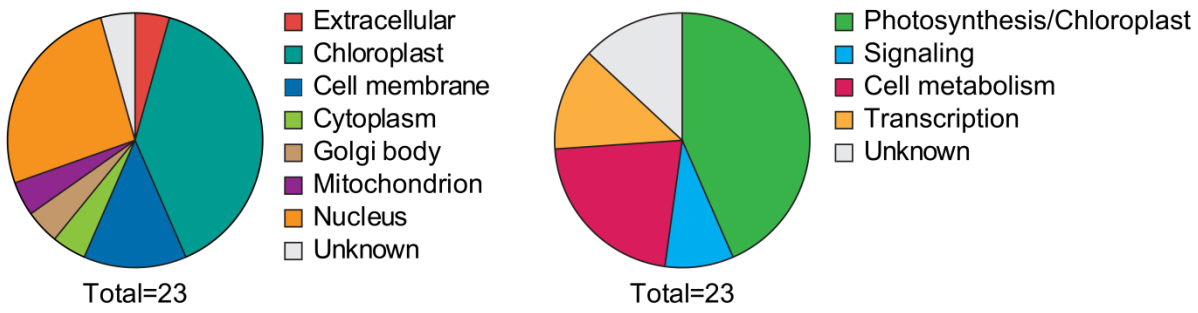


Figure 17. Subcellular localization (left) and functional categorization (right) of 23 validated *SOP* and *ENOP* genes. Genes were assigned separately to a subcellular localization and a biological function (color coded). This assignment was based on Gene Ontology annotations available at TAIR (http://www.Arabidopsis.org). The percentage of the total annotated genes within each class/sector is represented.

1.8 Light and PIF regulation of validated SOP and ENOP genes.

To begin to address whether the 23 validated regulator SOP and ENOP genes belong to the PIF-regulated transcriptional network that represses seedling deetiolation in the dark, we assessed their light and/or PIF transcriptional regulation by analyzing available microarray expression data of WT and *pifq* seedlings grown in continuous red light or darkness for 2 days (Leivar *et al.*, 2009). We considered PIF- (*pifq* dark VS WT dark) or Light- (WT light VS WT dark) regulated genes as those showing a statistical significant and at least a 1.5 fold change difference in gene expression between the indicated samples. In addition, we analyzed available ChIP-seq data of PIF1, 3, 4 and PIF5 (Pfeiffer *et al.*, 2014) to identify SOP and ENOP genes that have been described as PIF-bound genes. Compiled data are summarized in Table 2.

Table 2: PIF and light regulation of validated SOP and ENOP genes. For each validated SOP/ENOP gene, columns indicate the AGI gene number (AGI), the name assigned in the screening (screening name), the light and PIF regulation of gene expression (Light regulation and PIF regulation) (Leivar *et al.*, 2009), the PIFs that bind to the promoter region of the gene (PIF bound) (Pfeiffer *et al.*, 2014), and the organ affected by the suppressor or enhancer mutant phenotype (organ phenotype).

AGI	Screening name	Light regulation	PIF regulation	PIF bound	Organ Phenotype
AT1G53180	ENOP 139	Light repressed	PIF induced	PIF1PIF3PIF4	Cotyledon
AT5G50870	ENOP 158	Not regulated	Not regulated		Cotyledon
AT5G11730	ENOP 278	Not regulated	Not regulated		Cotyledon
AT3G13224	ENOP 47	No data	No data		Cotyledon / Hypocotyl
AT3G22840	ENOP 128	Light induced	PIF induced		Hypocotyl

AT1G73220	ENOP 269	Light repressed	PIF induced		Hypocotyl
AT3G58760	ENOP 122	Not regulated	Not regulated		Hypocotyl
AT4G03200	SOP 36	Not regulated	Not regulated	PIF1PIF4PIF5	Cotyledon
AT2G21280	SOP 38	Light induced	PIF repressed		Cotyledon
AT3G16250	SOP 69	Light induced	PIF repressed		Cotyledon
AT1G60940	SOP 86	Not regulated	Not regulated		Cotyledon
AT1G29470	SOP 116	Not regulated	Not regulated		Cotyledon
AT2G47760	SOP 52	Not regulated	Not regulated	PIF1	Cotyledon / Hypocotyl
AT3G08740	SOP 74	Not regulated	Not regulated		Cotyledon / Hypocotyl
AT1G18700	SOP 80	Not regulated	Not regulated		Cotyledon / Hypocotyl
AT3G13180	SOP 81	Not regulated	Not regulated		Cotyledon / Hypocotyl
AT5G39660	SOP 90	Not regulated	PIF repressed	PIF1	Cotyledon / Hypocotyl
AT5G59690	SOP 106	Not regulated	Not regulated		Cotyledon / Hypocotyl
AT4G32480	SOP 169	No data	No data	PIF1PIF3PIF4PIF5	Cotyledon / Hypocotyl
AT2G36000	SOP 119	Light induced	PIF repressed		Hypocotyl
AT5G10100	SOP 72	No data	No data		Hypocotyl
AT5G23010	SOP 153	Not regulated	Not regulated		Hypocotyl
AT4G22690	SOP 197	Light induced	PIF repressed	PIF4	Hypocotyl

According to table 2, we classified the different patterns of regulation in 4 categories:

Class I. Genes with no expression data.

ENOP 47, SOP 82 and SOP 169 do not have available microarray expression data as they are not represented with a probe in the ATH1 array. Therefore, further study by qPCR is needed to know whether they are light/PIF-regulated genes. Interestingly, one of them is a PIF-bound gene (SOP 169), indicating a possible role in the PIF-regulated transcriptional network.

Class II. PIF-bound genes.

ENOP 139, SOP 36, SOP 52, SOP 90, SOP 169 and SOP 197 are PIF-bound genes, suggesting that they are primary intermediaries of the PIF-signaling pathway. Two of them are PIF-repressed genes (SOPs 90 and 197), and ENOP 139 is a PIF-induced gene during deetiolation. SOP169 has no expression data as discussed in class I genes. The two remaining genes (SOP 36 and SOP 52) are not misregulated in 2 day-old dark-grown *pifq* seedlings, suggesting that PIF binding to their promoters does not trigger transcriptional regulation during seedling deetiolation. However, we reasoned that PIFs could still be regulating their expression in environmental conditions other than seedling deetiolation, such as in response to shade or under diurnal short day (SD) conditions (Leivar and Monte, 2014). Consequently, this subset of genes may have been identified as

regulators of photomorphogenesis during seedling deetiolation because the FOX screening involves ectopic and constitutive expression under the control of the 35S promoter (Figure 11).

Class III. PIF-regulated but not PIF-bound genes.

ENOP 128 and ENOP 269, causing an enhancement of the *pifq* photomorphogenic phenotype, are PIF-induced and light-regulated genes that are not PIF-bound. Conversely, SOPs 38, 69 and 119, causing a suppression of the *pifq* photomorphogenic phenotype, are PIF-repressed genes and light induced genes that are not PIF bound. These apparently contradictory PIF regulatory patterns suggest a more complex scenario than anticipated, in which our suppressor screen was predicted to identify PIF-induced genes causing suppressed photomorphogenic phenotypes (Figure 11). The data are consistent with a complex regulatory circuit acting downstream of the PIFs where the balance between PIF-regulated positive a negative signals determine the photomorphogenic response (Sentandreu *et al.*, 2011). Because these genes are PIF-regulated but not PIF-bound, they are likely not directly regulated by the PIFs and instead might be components of the downstream transcriptional cascade initiated by the PIFs.

Class IV. Genes that are not PIF-bound nor PIF-regulated.

Ten of the validated regulators are not PIF-regulated nor PIF-bound genes in 2 day-old dark-grown seedlings. On one hand, these genes might be PIF-bound or PIF-regulated genes in developmental conditions other than during seedling deetiolation, such as in response to shade or in short days (Leivar and Monte, 2014), as discussed earlier for class II genes. Alternatively, these genes might be components of light signaling pathways that regulate seedling deetiolation in a PIF-independent manner. These alternative regulatory components might include COP1, HY5, or hormone signaling components (Lau and Deng, 2010; Leivar and Monte, 2014).

2. Chloroplast function regulates photomorphogenic development.

2.1 PIFs repress the expression of light-induced genes involved in chloroplast biogenesis.

As explained above, we performed a suppressor screen of the constitutive photomorphogenic phenotype of *pifq* and we found a large number of suppressor mutants caused by overpression of genes coding for chloroplast proteins (Figure 17). Because of this result, we investigated PIF function regulating the expression of chloroplast proteins during seedling deetiolation. First, we obtained the list of genes coding for chloroplast proteins (http://ppdb.tc.cornell.edu/), and we analyzed their PIF and light transcriptional regulation by analyzing available expression data of 2 day-old dark- and light- grown WT and *pifq* seedlings (Leivar *et al.*, 2009). Interestingly, we observed that among the 1588 genes coding for chloroplast proteins with a representative probe in the ATH1 array, 33.5% of them (532 genes) were induced by light treatment (2 days light vs 2 days Dark) in a 2-fold statistically significant (SSTF) manner (Figure 20A). In contrast, a little percentage of these genes (1.38%) were repressed in response to light stimulus. Moreover, 33.1% of chloroplast proteins were coded by PIF-repressed genes (527 genes, Figure 20A) (Leivar *et al.*, 2009). This subset of PIF-repressed genes largely ovelaped with the subgroup of chloroplast proteins coded by light-induced genes (Figure 18A), in agreement with previous reports showing that PIF-regulated genes are also regulated by light (Leivar *et al.*, 2009). Next, we globally compared the expression level of these 1588 genes in 2 day-old dark-grown WT and *pifq* seedlings, and in WT seedlings grown for 2 days in the light (Figure 18B), and we determined that these genes coding for chloroplast proteins are induced in dark-grown *pifq* seedlings at similar levels than light-grown WT seedlings. Altogether, these results strongly suggest that genes coding for chloroplast proteins are repressed in the dark through PIF activity, leading to the maintenance of etioplasts, whereas genetic (in *pifq* seedlings) or light-induced (in WT seedlings) removal of PIFs leads to the induction of these genes and chloroplast biogenesis. These data confirm and extent previous analyses (Leivar *et al.*, 2009; Stephenson *et al.*, 2009).

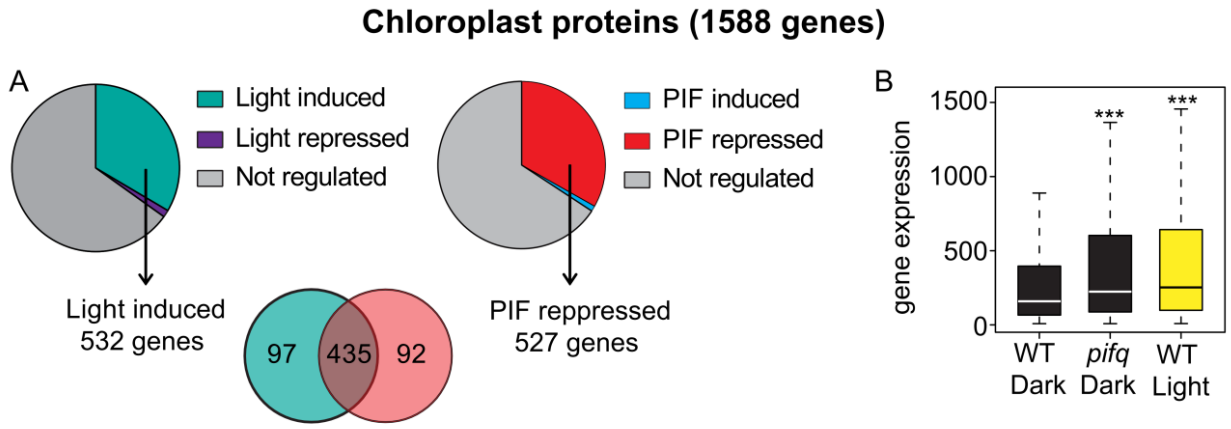


Figure 18. A large subset of chloroplast proteins is coded by PIF-repressed light-induced genes. (A) Top pie charts show the percentage of genes that code for chloroplast proteins that are induced, repressed or not regulated by light (right) in WT seedlings (2d light vs 2d Dark) or by the PIFs (left) in the dark (WT dark vs *pifq* dark). Differentially expressed genes were defined as genes responding by 1.5 fold change in a statistical significant (1.5FSS) manner. **(B)** Boxplot representation of microarray expression data of genes coding for chloroplast proteins in WT and *pifq* 2 days dark-grown seedlings (black) and WT 2 days light-grown seedlings (yellow), (*** p<0.001, Wilcoxom test).

2.2 Blocking chloroplast biogenesis suppresses the pifq cop-like phenotype.

Given our finding that overexpression of some chloroplast proteins in *pifq* seedlings supress the constitutive photomorphogenic development, we hypothesized that disruption of chloroplast protein stoichiometry might result in chloroplast dysfunction with an impact in photomorphogenesis. To test this, we grew WT and *pifq* for 3 days in the dark in the presence of lincomycin. Lincomycin is a drug that inhibits plastid 70S ribosomes, which results in the inhibition of plastid gene expression (PGE) and the blockage of chloroplast biogenesis during early seedling development (Sullivan and Gray, 1999a). First, we performed transmission electron microscopy of dark-grown WT and *pifq* seedlings in absence or presence of lincomycin in collaboration with Dolores Ludavid. We observed that lincomycin disturbed the ultrastucture of the etioplasts in WT seedlings, and of the partially developed chloroplasts in *pifq* seedlings, so that the prolamellar body and prothylakoid membranes were unrecognizable (Figure 19). These results allowed us to confirm the effect of lincomycin in plastid biogenesis in both genotypes.

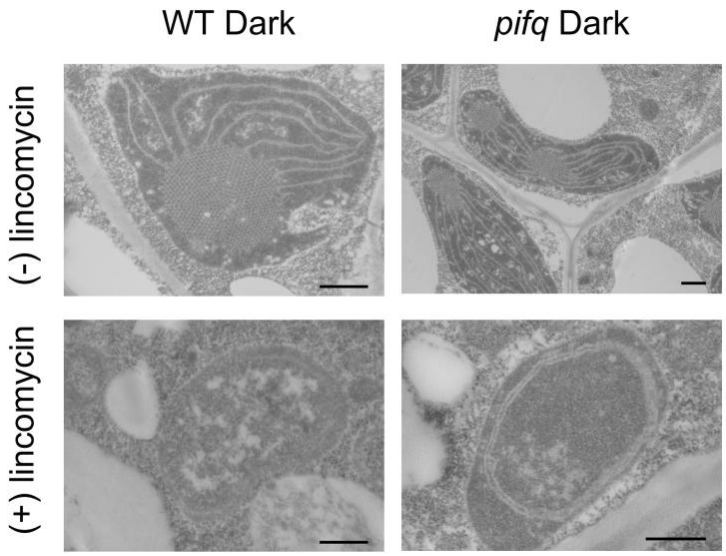


Figure 19. Lincomycin interferes with plastid development. Higher-magnification micrographs of samples prepared from 3 day-old light-grown WT and *pifq* seedlings in absence (top images) or presence (bottom images) of lincomycin. Representative etioplasts are shown for wild-type (left) and *pifq* (right) seedlings. Bars = 500nm.

Strikingly, parallel to the effect in blocking chloroplast development, dark-grown *pifq* seedlings in lincomycin showed partial suppression of the constitutive photomorphogenic phenotype (Figure 20A). Lincomycin induced hook formation and restored the cotyledon angle and expansion in *pifq* to levels similar to those exhibited by etiolated WT seedlings. Moreover, lincomycin also induced a modest but significant hypocotyl elongation in *pifq* seedlings (Figure 20B). In contrast, although lincomycin also suppressed etioplast structure in dark-grown WT seedlings (Figure 19), we did not observe a phenotypic effect of lincomycin in cotyledon angle, cotyledon area, or hypocotyl elongation, as these phenotypes already appeared closed, appressed and long respectively (Figure 20A, 20B). The only minor phenotypic effect of lincomycin observed in dark-grown WT seedlings was a slight closure of the hook (Figure 20A, 20B). These phenotypic analyses indicated that chloroplast dysfunction in *pifq* dark-grown seedlings supresses the photomorphogenic phenotype, and therefore, we can partially restore skotomorphogenic phenotype in *pifq* seedlings by adding lincomycin.

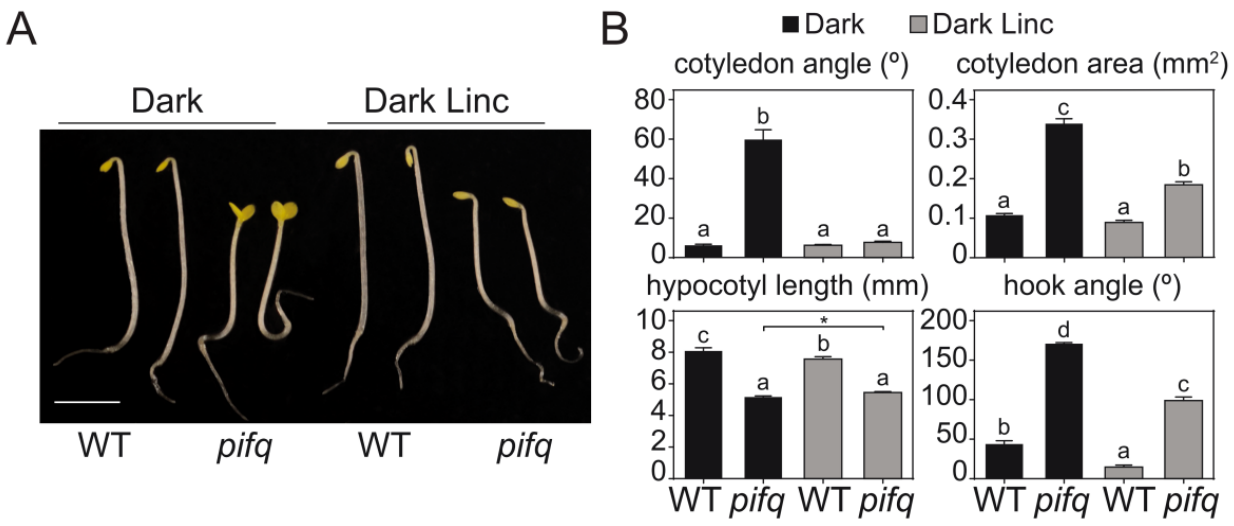


Figure 20. Blocking chloroplast biogenesis suppresses the *pifq* constitutive photomorphogenic phenotype. (A) Visual phenotype of wild type (WT) and *pifq* 3 day-old dark-grown seedlings in absence (left) or presence (right) of lincomycin (Linc). Bar = 2.5mm. **(B)** Quantification of cotyledon angle, cotyledon area, hypocotyl length and hook angle of WT and *pifq* seedlings grown as detailed in (A). Data are means ± SE of at least 40 seedlings. Different letters denote statistically significant differences among means (Tukey-b). Comparison of hypocotyl length between *pifq* and *pifq* Linc 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).

2.3 Lincomycin recovers the expression of pifq-misregulated genes in the dark through Retrograde Signaling.

It is well established that chloroplast dysfunctions produced by lincomycin affect the expression of nuclear genes through Retrograde Signaling (RS). Thus, we analyzed whether the lincomycin-mediated phenotypic suppression of the constitutive photomorphogenic phenotype of *pifq* could be due to changes in gene expression. For this, we analyzed lincomycin effect on *pifq* at the transcriptomic level. We performed a RNA-seq analysis of 3 day-old dark-grown *pifq* and WT seedlings grown in the presence or absence of lincomycin. First, we compared the lincomycin effect in gene expression in both WT and *pifq* genotypes, to define genes that were statistically significant two-fold (SSTF) regulated in response to lincomycin. In WT seedlings (WT Lincomycin VS WT), we identified 161 SSTF genes repressed and 73 SSTF genes induced by lincomycin. In *pifq* seedlings (*pifq* Lincomycin VS *pifq*), we identified a larger amount of lincomycin-responsive genes, including a subset of 384 lincomycin-repressed and a subset of 581 lincomycin-induced genes (Figure 21A). To correlate these lincomycin-induced changes in nuclear gene expression with the possible activation of retrograde signaling, we compared our lincomycin-responsive genes with

genes described previously as RS-responsive. RS-responsive genes was previously defined as those genes that are regulated by the plastid biogenesis inhibitors norflurazon and lincomycin in the light (Woodson *et al.*, 2013). We observed a large overlap between previously described RS-repressed genes and our list of lincomycin-repressed genes in both WT (75%) and *pifq* (81%), and between previously described RS-induced genes and our list of lincomycin-induced genes in WT (65%). Intriguingly, only 37% of lincomycin-induced genes in *pifq* in our experiment overlapped with previously described RS-induced genes (Figure 21A). The large overlap between our lincomycin-responsive genes and the previously defined RS-responsive genes, at least the repressed subset, indicated that retrograde signaling is activated in our dark conditions in the presence of lincomycin. Moreover, we measured the expression of *LHCB1.4* by qRT-PCR in the same conditions as in the RNA-seq experiment, as *LHCB1.4* is reported to be strongly downregulated in response to retrograde signaling (Koussevitzky *et al.*, 2007). Consistent with the proposed RS function in our conditions, we observe a strong downregulation of *LHCB1.4* in *pifq* dark-grown seedlings treated with lincomycin (Figure 21B).

A

	SSTF Lincomycin repressed	SSTF Lincomycin induced
WT dark grown	161 genes (75%)	73 genes (65%)
pifq dark grown	384 genes (81%)	581 genes (37%)

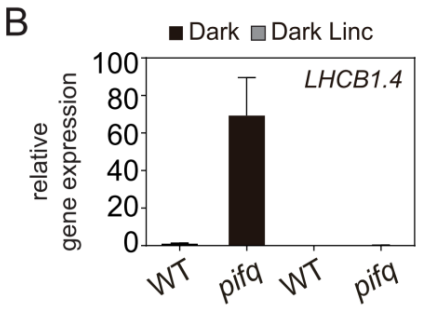


Figure 21. Retrograde Signaling is activated in lincomycin-treated dark-grown seedlings. (A) Table shows the number of lincomycin-responsive genes in a twofold change statistical significant (SSTF) manner, in wild type (WT) (top) and *pifq* (bottom) dark-grown seedlings. Percentage represents the overlap between identified lincomycin-regulated genes in our RNAseq experiment, excluding genes that are not present in ATH1 array, and genes previously reported as Retrograde Signaling-regulated (Woodson *et al.*, 2013) **(B)** Expression of *LHCB1.4* analyzed by quantitative RT-PCR in WT and *pifq* 3-day-old dark (black) and dark plus lincomycin (grey) seedlings. Values were normalized to *PP2A* and expression levels are shown relative to WT Dark. Data are the means ± SE of biological triplicates.

To further understand RS function in the suppression of the *pifq* constitutive photomorphogenic phenotype, we analyzed in more detail the transcriptional response of PIF-regulated genes to lincomycin. First, we compared gene expression of WT and *pifq* seedlings in the dark, and identified 521 SSTF PIF-repressed and 1826 SSTF PIF-induced genes (Figure 22). Of the 521 SSTF PIF-repressed genes, 354 genes (67.9%) were statistically and significantly (SS)

repressed in response to lincomycin in *pifq* mutants (*pifq* VS *pifq* Linc), 38 genes were SS induced (7.29%), and 129 (24.76%) did not respond to lincomycin. In addition, of the 1826 SSTF PIF-induced genes, 793 genes (43.4%) were SS induced in response to lincomycin, 14 genes were repressed (0.76%), and 1019 (55.80%) were not lincomycin-regulated (Figure 22). These results showed that lincomycin strongly regulates a subset of PIF-regulated genes mainly in the same direction as the PIFs, i.e. by repressing the expression of PIF-repressed genes and by inducing the expression of PIF-induced genes. Because lincomycin tends to reverse the expression of these genes in *pifq* to WT levels, these genes were called 'PIF- repressed reversed' and 'PIF- induced reversed' genes, respectively.

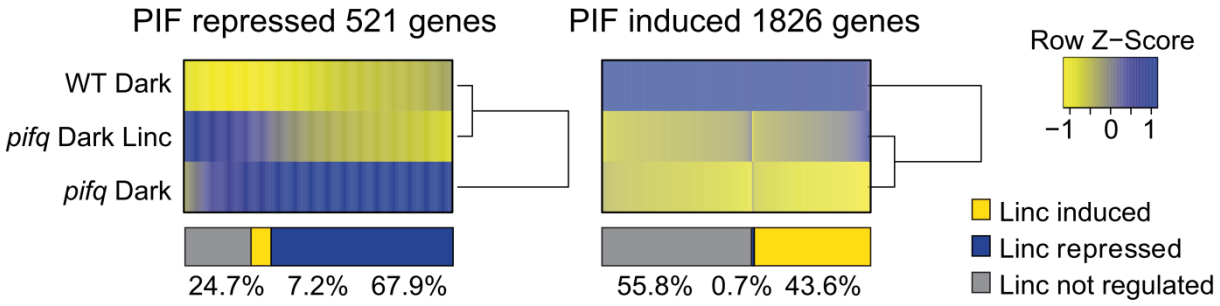


Figure 22. Lincomycin regulates PIF-regulated genes in the same direction as PIFs. Two-dimensional-cluster diagram depicting expression levels of 521 PIF-repressed (left) and 1826 PIF-induced (right) SSTF genes in the dark in the absence (Dark) or presence (Dark Linc) of lincomycin. SSTF: genes whose expression changed statistically significantly and by at least twofold.

Next, we calculated the percentage of lincomycin reversion for each of the PIF-regulated reversed genes. To calculate this percentage, we first calculated the "lincomycin effect". This parameter was defined as the difference in expression between dark-grown *pifq* with lincomycin (*pifq* Linc) and WT without lincomycin (WT), relative to the difference in expression between dark-grown *pifq* and WT without lincomycin (lincomycin effect = *pifq* Linc – WT / *pifq* - WT). Then, to calculate the percentage of lincomycin reversion we applied the formula (1-lincomycin effect) x 100. Values of lincomycin reversion similar to 100% indicate that expression of the PIF-regulated gene was equal to the WT dark values in *pifq* dark-lincomycin seedlings. For the 354 PIF-repressed reversed genes, 331 genes (93%) reverted the expression in response to lincomycin more than 50%, and 270 genes (76.7%) reverted more than 75% (Figure 23A). For the 793 PIF-induced reversed genes, only 199 genes (25%) reverted the expression more than 50% (Figure 23B). These results showed that lincomycin strongly restores the *pifq* transcriptome to WT dark, in agreement with the suppression of *pifq* photomorphogenic phenotype. We also conclude that lincomycin had a stronger

effect reverting *pifq* gene expression of PIF-repressed genes, which are overexpressed in the *pifq*, than PIF-induced genes.

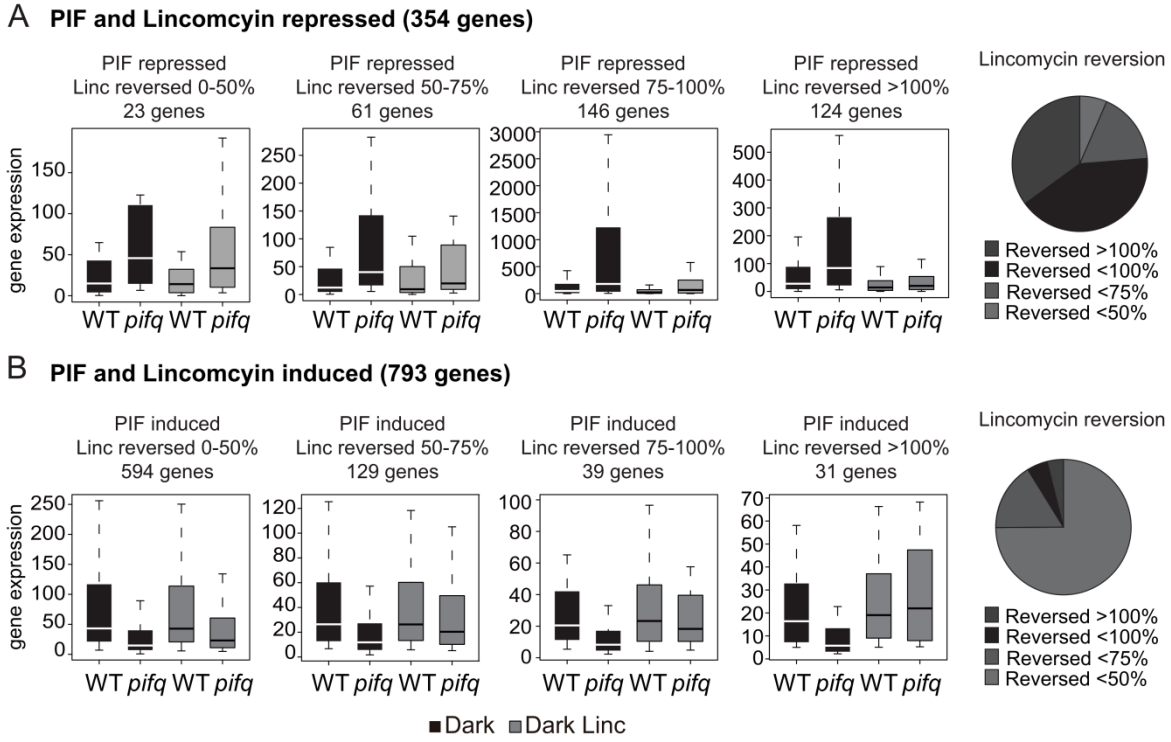


Figure 23. Lincomcyin reversion of *pifq* misregulated transcriptome. Boxplot representation of mRNA levels in 3-day-old dark-grown wild type (WT) and *pifq* mutants in the absence (black) or presence (grey) of lincomycin (Linc) (four left graphs), and pie chart of genes sorted by lincomycin reversion percentage (right), of the 354 PIF and lincomycin repressed genes **(A)** and of the 793 PIF and lincomycin induced genes **(B)**. In the boxplots, genes were sorted by the percentage of lincomycin reversion: less than 50% (left), between 50 and 75% (middle left), between 75 and 100% (middle right) or more than 100% (right).

Importantly, retrograde signals did not affect the expression of well-described PIF-target genes that had been previously related to growth (Figure 24), indicating that the regulation of photomorphogenesis by the convergence of PIF-RS pathways may involve other molecular mechanism downstream of PIF proteins than those previously reported.

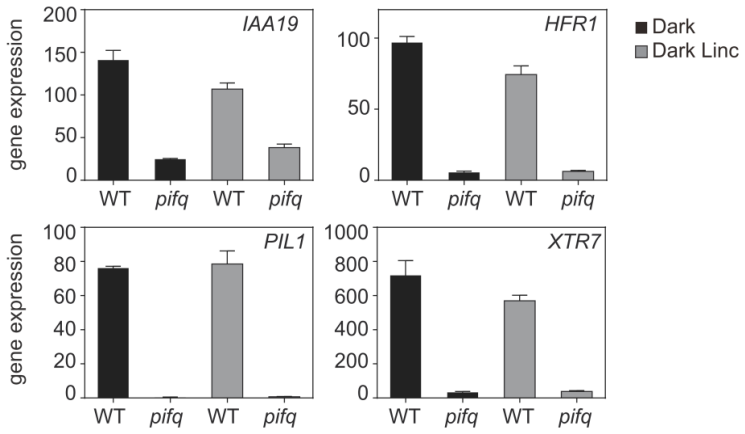


Figure 24. Expression of PIF target genes in response to lincomycin. Bar graph of RNA-seq data showing the expression of *IAA19, HFR1, PIL1* and *XTR7* in WT and *pifq* mutants grown for three days in the dark in presence (grey) or absence (black) of lincomycin. Data correspond to biological triplicates, and bars indicate SE.

2.4 Retrograde Signaling prevents photomorphogenesis in light-grown seedlings by repressing the expression of Light induced/PIF repressed genes.

Because of the compelling overlap between PIF- and RS-regulated transcriptional networks in the dark, we next studied this interplay in the light, by analyzing the effect of lincomycin in 3 day-old light-grown WT seedlings. Interestingly, we observed that the phenotype of light-grown WT seedlings on lincomycin resembled that of a dark-grown WT seedling (Figure 25A). Quantification of these phenotypes showed that lincomycin completely restored the cotyledon angle to the WT dark-grown levels, while significantly suppressing the cotyledon area and inducing hypocotyl elongation (Figure 25B). Together, these results showed that, similar to dark-grown *pifq* (Figure 20), lincomycin repress photomorphogenesis in WT light-grown seedlings, indicating that retrograde signaling is an important mechanism that regulates photomorphogenic development in light environments.

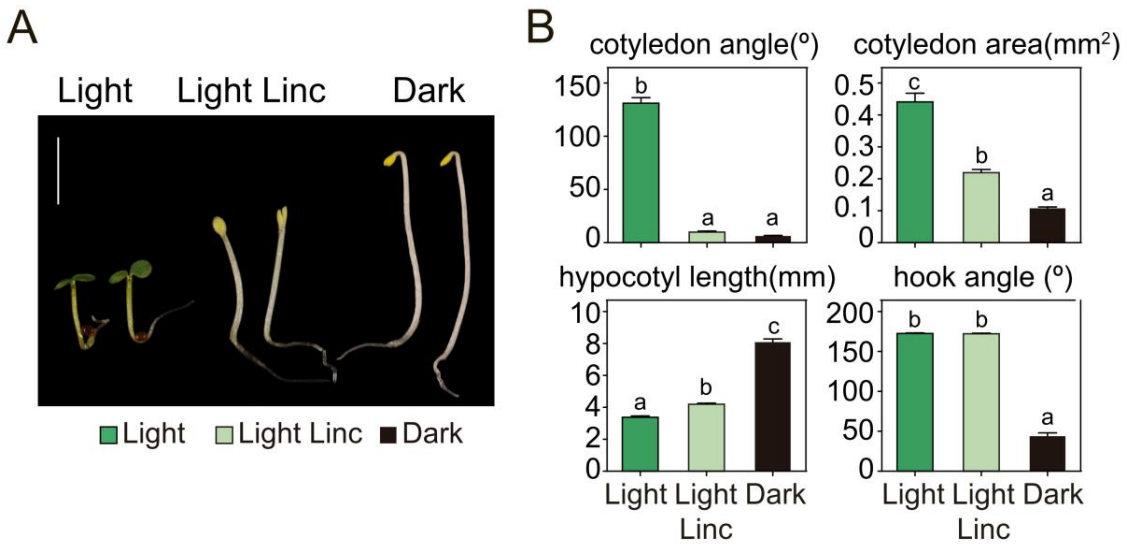


Figure 25: Retrograde signaling prevents photomorphogenesis in light-grown WT seedlings. (A) Visual phenotype of WT seedlings grown for 3 days in the light in the absence (left) or presence (middle) of lincomycin (Linc), or for 3 days in darkness (right). Bar = 2.5mm. **(B)** Quantification of cotyledon angle, cotyledon area, hypocotyl length and hook angle of WT seedlings grown as detailed in A. Data are means ± SE of at least 40 seedlings, and different letters denote statistically significant differences among means (Tukey-b).

Because our RNAseq experiment suggested that lincomycin repressed the constitutive photomorphogenic phenotype of *pifq* dark-grown seedlings by reversing the expression of PIF-regulated genes, we next addressed whether the photomorphogenic suppression of WT ligh-grown seedlings was also due to the transcriptional reversion of the light-induced PIF-repressed transcriptional netwok. To study the interplay between the light/PIF-regulated and the RS-regulated transcriptional networks in the light, we performed a comparison between previously described PIF and light regulated genes (Leivar *et al.*, 2009), and genes previously reported as RS-upregulated (660 genes) or RS-downreagulated (978 genes) (Woodson *et al.*, 2013). As described elsewhere (Leivar *et al.*, 2009), this comparison first showed that PIF and light-regulated genes extensively overlap (Figura 26). Interestingly, of the 499 genes defined as PIF-repressed and light-induced, a 68.7% (343 genes) were also defined as RS-downregulated genes (Figure 26, left panel). In contrast, only 6% (19 genes) of PIF-induced and light-repressed genes were RS-upregulated genes (Figure 26, right panel). This smaller overlap between PIF-induced/light-repressed and RS-induced genes was consistent with the minor effect of lincomycin in reverting expression of PIF-induced genes in dark-grown *pifq* seedlings (Figure 22, 23).

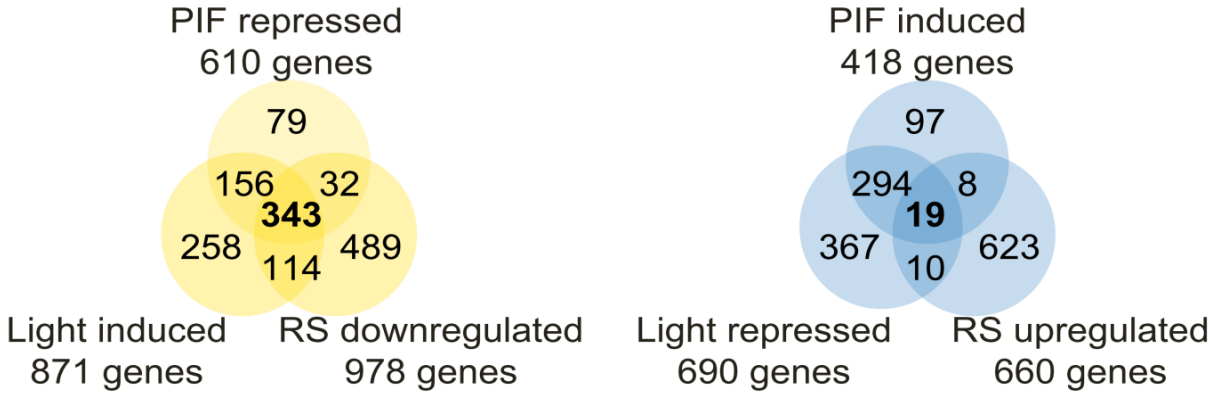


Figure 26. PIF-repressed genes targeted by Retrograde Signaling are mainly light induced genes. Venn diagrams showing the overlap between Retrograde Signaling (RS)-downregulated genes described previously (Woodson *et al.*, 2013) and genes described as light-induced and PIF-repressed (Leivar *et al.*, 2009) (left), and between genes described as RS-upregulated (Woodson *et al.*, 2013), light-repressed and PIF-induced (Leivar *et al.*, 2009) (right).

In addition, the extensive overlap between RS-downregulated genes and light-induced/PIF-repressed genes indicates that retrograde signaling represses photomorphogenesis by repressing the expression of this light-induced transcriptional network regulated by PIFs. Accordingly, global expression analysis of these 343 coregulated genes (referred as "gene set PIF-RS" showed that the global expression profile of WT seedlings grown in the light and on lincomycin was more similar to WT-dark than to WT-light (Figure 27).

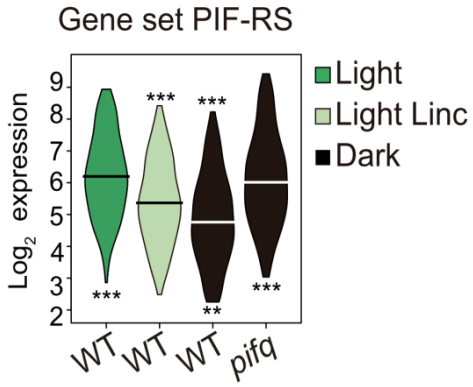


Figure 27. Expression data of 343 coregulated genes (gene set PIF-RS). Violin plot of microarray expression data in 5 day-old light-grown WT in absence (Light, dark green) or presence (Light Linc, light green) of lincomycin, and in 2 day-old dark-grown WT and *pifq* (Dark, black). Statistically significant differences by heteroscedastic t test between the light-grown WT and each of the other samples are indicated in the upper part of the plot, whereas statistically significant differences between light and lincomycin-grown WT and each of the other samples are indicated in the lower part. Data were obtained from GSE5770 (Koussevitzky *et al.*, 2007) and GSE17159 (Leivar *et al.*, 2009).

2.5 PIFs and retrograde signaling represses a specific subset of genes to coordinate the developmental program.

Because lincomycin reverted the photomorphogenic phenotype and a subset of light-induced/PIF-repressed genes both in the dark (in *pifq* seedlings) and in the light (in WT seedlings), we next tested whether the genes responding to lincomycin/RS in the dark were the same as those responding to RS in the light. For this, we first analyzed the overlap between the defined subset of 343 PIF-RS genes in the light (Figure 26), and the subset of PIF-repressed genes that are repressed in response to lincomycin in the dark (Figure 22), but considering only those that are represented in the ATH1 array (317 out of 354 genes). Comparison of both subsets showed a big overlap (Figure 28A) indicating that, as at the phenotypic level, lincomycin produces similar molecular changes in WT light- and *pifq* dark-grown seedlings.

Next, to obtain additional evidence that the molecular effect of lincomycin was similar in the dark and in the light, we studied the expression profile of the subsets of genes defined in Figure 24 abd 27 (PIF repressed lincomycin repressed in darkness, and PIF-RS). For this, we analyzed RNAseq data reported here together with available microarray expression data (Koussevitzky *et al.*, 2007; Leivar *et al.*, 2009). As a result of this analysis, we observed that globally both subset of genes behaved similarly. In the RNAseq experiment performed in the dark, both subsets were PIF-repressed (upregulated in *pifq*) and lincomycin reversed (Figure 28B). In the microarray experiments performed in the light, both subsets were light-induced and downregulated in response to lincomycin (Figure 28B). Together, these data describe a set of light-induced PIF-repressed genes that are strongly repressed by lincomycin-induced RS both in the dark and in the light. The expression profile of this subset of genes strongly correlates with the photomorphogenic development of the seedling: they are upregulated in photomorphogenic seedlings (WT-light or *pifq*-dark), and are repressed in skotomorphogenic seedlings (WT-dark) or in seedlings with activated RS by lincomycin.

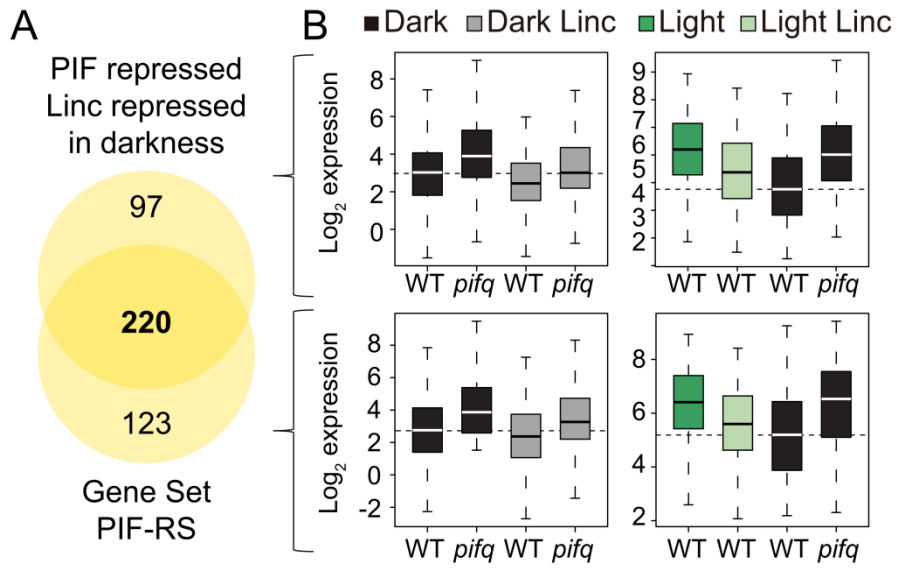


Figure 28. PIF-repressed lincomycin-repressed genes in the dark extensively overlap with the genes in the PIF-RS gene set. (A) Venn diagram showing overlap between the 354 PIF- and lincomycin (linc)-repressed gene set in the dark defined in figure 22, and the 343 genes defined as 'gene set PIF-RS' (Figure 26). Only 317 out of the 354 PIF- and lincomycin-reversed gene set defined by our RNAseq are present in the ATH1 array, and were used here for the comparison. **(B)** Boxplot representation of the transcript levels of the 354 PIF- and lincomycin-repressed genes in darkness (top), and the 343 genes in gene set PIF-RS (bottom). Data show the RNAseq expression in 3 day-old dark-grown wild type (WT) and *pifq* seedlings in the absence (dark) or presence (grey) of lincomycin (Linc) (left), and the microarray expression in 5 day-old light-grown WT in absence (dark green) or presence (light green) of lincomycin compared to 2 day-old dark-grown WT and *pifq* (black) (right). The median value in WT dark en each boxplot is represented by a black dashed line. Data for the 343 PIF-RS gene set was obtained from GSE5770 (Koussevitzky *et al.*, 2007) and GSE17159 (Leivar *et al.*, 2009).

Next, as PIF-RS genes are PIF repressed in the dark, we wondered whether the regulation of PIFs is direct. We analyzed available ChiP-seq data of the four PIFs (PIF1, 3, 4 and 5) (Pfeiffer *et al.*, 2014), and we observed that only 13% of PIF-RS genes are PIF bound genes (Figure 29A), indicating that PIF regulation of these set of genes is mainly indirect. Therefore, these data suggest that there must be an intermediary factor acting downstream of PIF transcription factors to regulate the expression of these genes. Moreover, to begin to categorize their function, we analyzed the subcelular localization of the gene set PIF-RS and we observed a clear enrichment of genes that code for chloroplast proteins (Figure 29B). This result correlates with our previous analysis where we showed that approximately 30% of chloroplast proteins are encoded by PIF-repressed and light-induced genes (Figure 18), and also with current data showing that retrograde signaling strongly represses the expression of photosynthetic associated genes (Woodson and Chory, 2008).

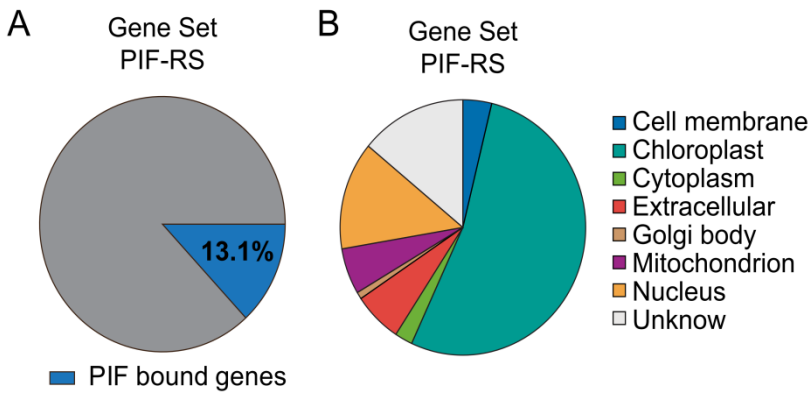


Figure 29. Genes in the 'PIF-RS set' are mainly not PIF-bound and are enriched in genes coding for chloroplast proteins. (A) Percentage of PIF-RS genes, defined in figure 26, that have been reported previously as PIF bound genes (Pfeiffer *et al.*, 2014). **(B)** PIF-RS genes were assigned to a separate subcelular localization (color coded). This assignment was based on Gene Ontology annotations available at TAIR (http://www.Arabidopsis.org). The percentage of the total annotated genes within each class/sector is represented.

2.6 Repression of photomorphogenesis by lincomycin-induced retrograde signaling is mediated by GUN1

To gain insight into the molecular mechanisms underlying the effect of retrograde signals in photomorphogenic development, we studied the role of ABI4 and GUN1, described as major regulators of the RS pathway (Koussevitzky *et al.*, 2007). For this purpose, we analyzed the phenotypes of WT, *abi4* and *gun1* mutants grown for 3 days in the light in the presence or absence of lincomycin. As shown before, WT seedlings grown in medium containing lincomycin showed suppression of the photomorphogenic phenotype, including inhibition of cotyledon aperture, and partial elongation of hypocotyls (Figure 30). Interestingly, *gun1* mutants in presence of lincomycin did not show repression of photomorphogenic development because the cotyledon aperture was not inhibited, and hypocotyl did not elongate in response to lincomycin. In contrast, *abi4* responded to lincomycin, by displaying closed cotyledons and elongated hypocotyls, although this elongation was relatively reduced compared to WT (Figure 30A, 30B).

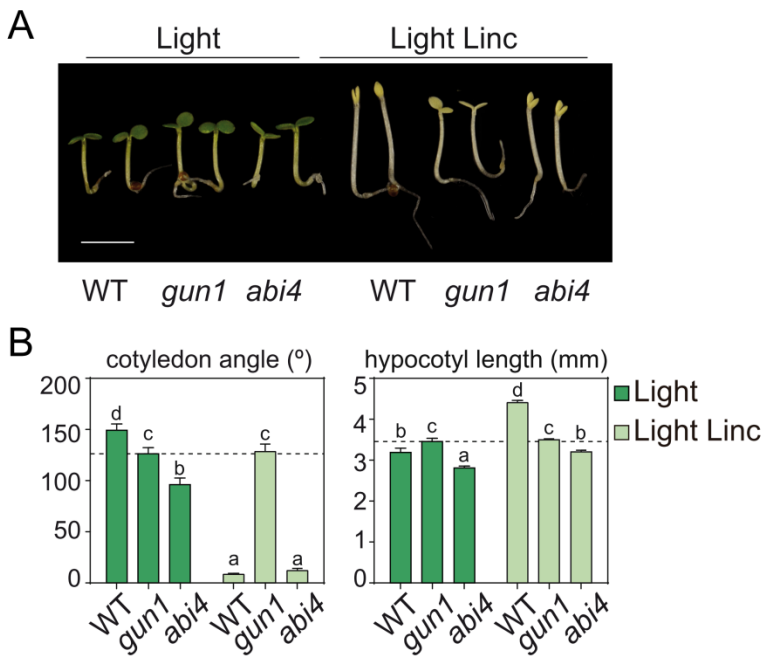


Figure 30. GUN1 and ABI4 function in retrograde signaling regulation of photomorphogenesis. (A) Visual phenotype of 3 day-old light-grown WT, *abi4* and *gun1* mutants in the absence or presence of lincomycin (Linc). Bar = 2.5mm. **(B)** Quantification of cotyledon angle (left) and hypocotyl length (right) of WT, *abi4* and *gun1* seedlings grown as detailed in A. Data are means ± SE of at least 50 seedlings, and different letters denote statistically significant differences among means (Tukey-b).

In order to study the molecular phenotypes of *abi4* and *gun1* mutants in response to lincomycin, we analyzed published microarray expression data of the 343 PIF-RS genes in 5 day-old light-grown WT, *abi4* and *gun1* in absence or presence of lincomycin (Koussevitzky *et al.*, 2007). Consistent with the morphological phenotypes, the expression of the 343 PIF-RS genes was not globally affected by mutations in ABI4 and GUN1 in normal light conditions. However, whereas in lincomycin-light grown seedlings the expression was strongly downregulated in WT and *abi4* backgrounds, this response was not observed in the *gun1* mutant (Figure 31). Hence, this transcriptomic analysis correlates with the phenotypic data, where GUN1 but not ABI4 has a major role in mediating RS-suppression of photomorphogenic development. From these collective data, we concluded that GUN1, but likely not ABI4, mediates the lincomycin-induced RS that inhibits the expression of light-induced/PIF-repressed genes, thereby repressing seedling photomorphogenesis in the light in response to chloroplast dysfunction.

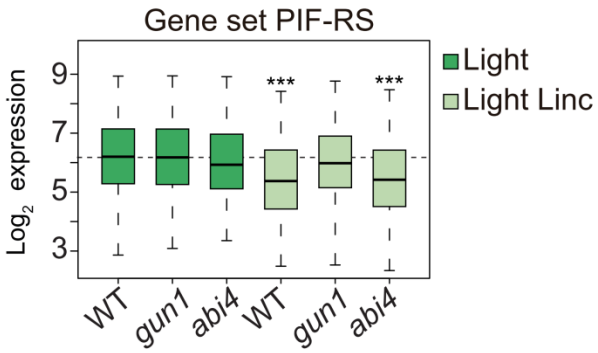


Figure 31. GUN1 and ABI4 function in transcriptional regulation of PIF-RS gene set. Boxplot representation of microarray data showing the global expression of the Gene Set PIF-RS in 5 day-old light-grown WT, *abi4* and *gun1* mutants in the absence (dark green) or presence (light green) of lincomycin. Statistically significant differences from WT light by heteroscedastic t test are indicated. Data obtained from GSE5770 (Koussevitzky *et al.*, 2007).

2.7 PIF and RS converge on GLK1 to repress chloroplast biogenesis and photomorphogenesis.

Given that i) PIFs are not directly regulating the subset of PIF-RS genes (Figure 29A), and ii) *GUN1* does not appear to be a PIF-bound gene (Pfeiffer *et al.*, 2014) and is not a PIF-regulated gene, phytochrome-mediated-light and plastid RS signaling must converge downstream of GUN1 and the PIFs, likely through co-regulation of one or more common target genes. Interestingly, analysis of DNA binding motifs revealed that the 'Gene-Set PIF-RS' was significantly enriched in the putative GLK (GOLDEN2-LIKE) binding site CCAATC (Waters *et al.*, 2009) (Figure 32).

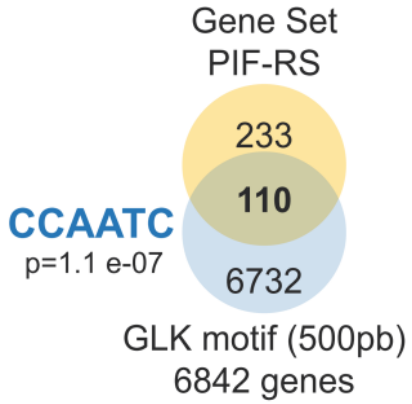


Figure 32. 'Gene Set PIF-RS' is significantly enriched in putative GLK binding sites. Venn diagram showing the overlap between PIF-RS genes and the 6842 genes in the genome that have the GLK1 binding motif within the 500pb upstream regulatory region. GLK putative binding motif (CCAATC) was defined in (Waters *et al.*, 2009).

GLK1 is a transcription factor that has been proposed to act downstream of GUN1 when retrograde signaling is activated by addition of norflurazon, an inhibitor of carotenoid biosynthesis that blocks chloroplast biogenesis, or by the genetic removal (*ppi2-1* mutant) of proteins involved in plastid import protein (Kakizaki *et al.*, 2009). Moreover, GLK1 has been described as an important transcriptional activator of photosynthesis associated genes (pHANG) (Waters *et al.*, 2009), consistent with a role of GLK1 in regulating the expression of our subset of PIF-RS genes that are vastly enriched in genes coding for chloroplast proteins (Fig 29B). These facts together with the observation that *GLK1* is a PIF-repressed gene directly targeted by PIFs in the dark (Oh *et al.*, 2012; Pfeiffer *et al.*, 2014; Song *et al.*, 2014) (Figure 33), makes *GLK1* as an strong candidate to integrate both PIF and RS signals to promote chloroplast biogenesis and photomorphogenesis.

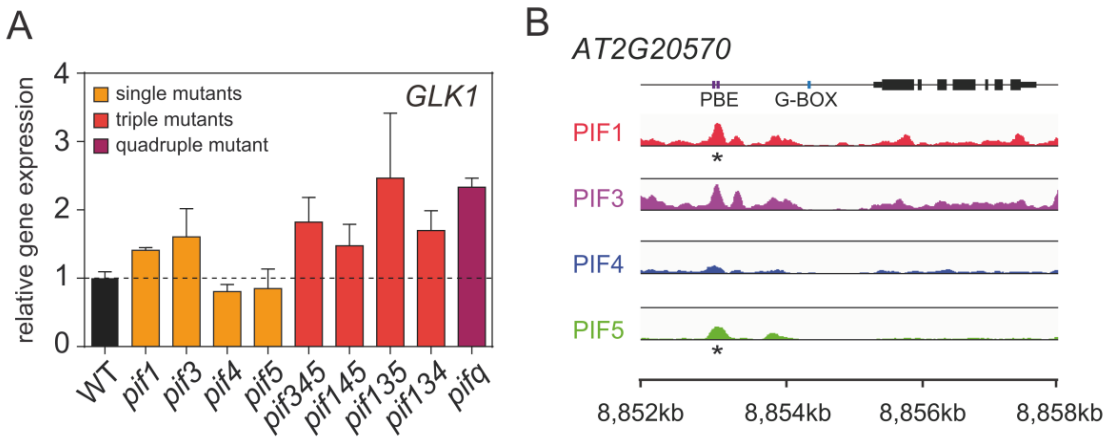


Figure 33. Compiled ChIP–seq and RNA–seq data identify *GLK1* as direct target of PIF transcriptional regulation. (A) Mean expression value of *GLK1* in dark-grown single *pif1*, *pif3*, *pif4*, and *pif*5 mutants, in all the triple *pif* mutant combinations, and in *pifq*. Data are expressed relative to the WT set at one (dashed line). Data are RNA-seq data from (Pfeiffer *et al.*, 2014). **(B)** Visualization of ChIP-seq data in the genomic region encompassing the *GLK1* locus. Identified significant binding sites are indicated by an asterisk below the pile-up ChIP-seq tracks, and extends to 200bp around the centered peak summit defined as the binding-peak maximum. G- and PBE-box motifs in the promoter are indicated. Data obtained from (Pfeiffer *et al.*, 2014). Additionally, *GLK1* was defined as a PIF4-bound gene by ChIP-qPCR (Song *et al.*, 2014) and in a ChIP-seq experiment (Oh *et al.*, 2012).

To analyze the transcriptional regulation of *GLK1* by light and retrograde signaling, *GLK1* expression was first analyzed by quantitative RT-PCR in 3 day-old dark-grown WT and *pifq* seedlings, and in 3 day-old light-grown WT, *abi4* and *gun1* seedlings, either in the presence or in the absence of lincomycin. As expected, we observed that *GLK1* is repressed by PIFs in the dark, because *pifq* mutants had elevated expression in the dark, and induced by light (Oh and Montgomery, 2014), which is consistent with derepression due to phy-induced PIF removal (Figure 34). Furthermore, the expression was strongly repressed in either WT or *pifq* dark- or WT light-

grown seedlings in response to lincomycin. Strikingly, lincomycin did not repress the expression of GLK1 in light-grown *gun1* mutants, an effect not observed in *abi4* mutants (Figure 34), which is in agreement with our previous results that show that GUN1, but not ABI4, plays a relevant role repressing photomorphogenic development after lincomycin addition (Figure 30). Together, these data show that dark signals transduced by the PIFs and retrograde signals transduced by GUN1 converge to repress the expression of *GLK1*.

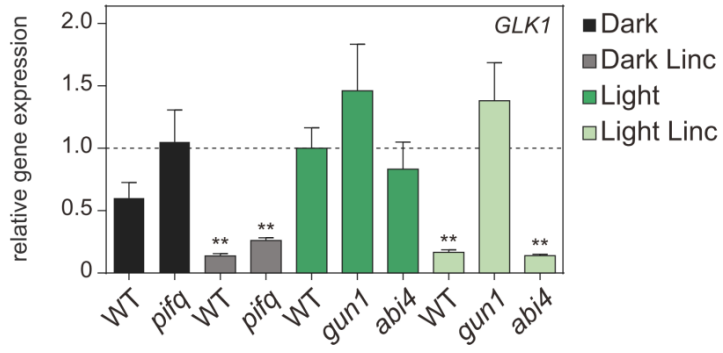


Figure 34. *GLK1* is repressed in the dark and by retrograde signaling through PIFs and GUN1, respectively. Expression of *GLK1* was analyzed by quantitative RT-PCR in 3-day-old seedlings grown in the dark (Dark), dark plus lincomycin (Dark Linc), continuous white light (Light) and continuous light plus lincomycin (Light Linc). Values were normalized to *PP2A* and expression levels are shown relative to wild type (WT) light, set at unity (dashed line). Data are the means ± SE of biological triplicates and asterisks indicate statistically significant differences from light-grown WT seedlings by Student's *t* test.

Because PIF and RS signaling pathways converge to regulate *GLK1*, we next studied the PIF and RS regulation of the GLK1-regulated transcriptional network. For this, we analyzed microarray expression data of GLK1-regulated genes in 5 day-old light-grown WT in absence or presence of lincomycin (Koussevitzky *et al.*, 2007), and 2 day-old dark-grown WT and *pifq* (Leivar *et al.*, 2009). The subset of GLK1-regulated genes was defined previously (Waters *et al.*, 2009), and because these genes were mostly GLK1-upregulated, it was concluded that GLK1 is a transcriptional activator. Global analysis of the GLK1-upregulated genes in response to light and retrograde signals showed that their expression pattern correlated very well with the *GLK1* expression pattern defined here (Figure 34), further indicating that the function of GLK1 as a transcriptional regulator is repressed in the dark and in response to lincomycin (Figure 35A). Moreover, GLK1-upregulated genes were downregulated and expressed at similar levels in seedlings with skotomorphogenic development (WT dark-grown, and WT and *abi4* grown on lincomycin+light), and upregulated in seedlings with photomorphogenic phenotype (*pifq* dark-grown, WT, *abi4* and *gun1* light-grown, and *gun1* grown on lincomycin+light) (Figure 35A). The

coincidence between the expression pattern of GLK1-upregulated genes and the seedling phenotype suggests that GLK1 is a key regulator of seedling photomorphogenesis. In accordance with this potential new role of GLK1 regulating photomorphogenesis, together with the observed link between the expression pattern of PIF-RS genes and photomorphogenic development (Figure 25, 27, 30, 31), we observed a strong enrichment of PIF-RS genes among GLK1-upregulated genes (Figure 35B).

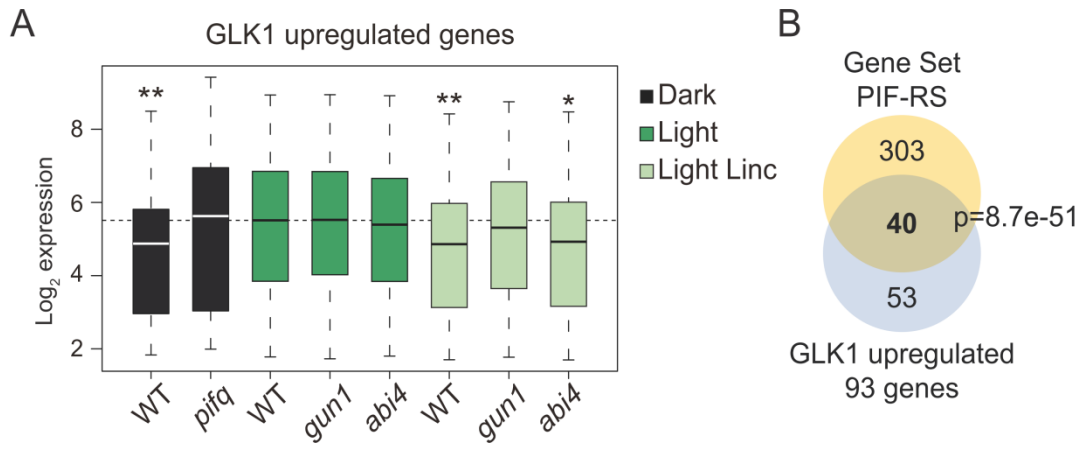


Figure 35. GLK1-upregulated genes are significantly enriched in PIF-RS genes. **(A)** Boxplot representation of transcript levels of a set of 93 genes previously defined as GLK1-induced (GLK1 upregulated) (Waters *et al.*, 2009)that have a corresponding probe in the ATH1 array. Data are from microarray experiments that included 2 day-old dark-grown WT and *pifq* (black), and 5 day-old light-grown WT, *gun1* and *abi4* in the absence (dark green) or presence (light green) of lincomycin. Statistically significant differences from WT light by heteroscedastic t test are indicated. Data obtained from GSE5770 and GSE17159 (Koussevitzky *et al.*, 2007; Leivar *et al.*, 2009). **(B)** Venn diagram showing the overlap between the 343 genes in gene set PIF-RS and the 93 genes upregulated by GLK1 (Waters *et al.*, 2009) that have a corresponding probe in the ATH1 array. P-value corresponds to hypergeometric statistical test to assess the significance of the number of PIF-RS genes in the GLK1 upregulated gene set compared to the total population of genes represented on the ATH1 array.

2.8 GLK1 induces photomorphogenic development.

Given that our data suggest that light and RS signals converge on *GLK1* expression to regulate photomorphogenic development, we tested this possible role of GLK1 in early seedling development by analyzing the phenotype of *glk1* mutant seedlings grown in the light for 3 days. Interestingly, *glk1* mutants showed a hyposensitive phenotype in continuous white light, with closed cotyledons and longer hypocotyls (Figure 36), confirming that *GLK1*, a light-induced gene, induces photomorphogenesis in normal light conditions.

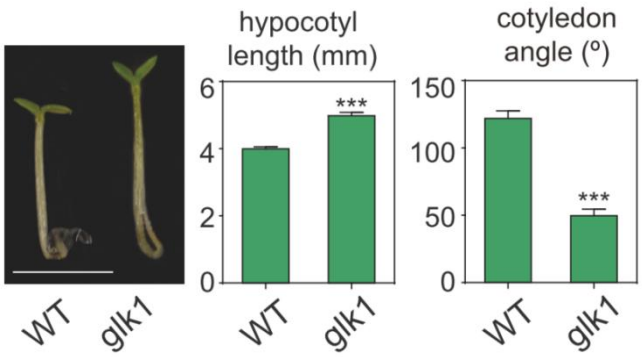


Figure 36. GLK1 induces photomorphogenesis in light conditions. Visual phenotype (left), quantification of cotyledon angle (middle) and hypocotyl length (right) of 3 day-old light-grown WT and *glk1* mutants. Bar = 2.5 mm. Data are means ± SE of at least 40 seedlings and asterisks indicate statistically significant differences from light-grown WT seedlings by Student's *t* test.

Next, we studied whether lincomycin-induced *GLK1* downregulation was important to repress seedling photomorphogenesis. To address this question, we measured the photomorphogenic development of *GLK1OX* lines, where *GLK1* is expressed constitutively under the control of the strong CaMV35S promoter, in plates containing lincomycin. Interestingly, in contrast to WT, *GLK1OX* seedlings were resistant to the lincomycin treatment as they were not able to close cotyledons and elongate hypocotyls (Figure 37). This result strongly suggests that *GLK1* downregulation is necessary to suppress photomorphogenic development in response to lincomycin.

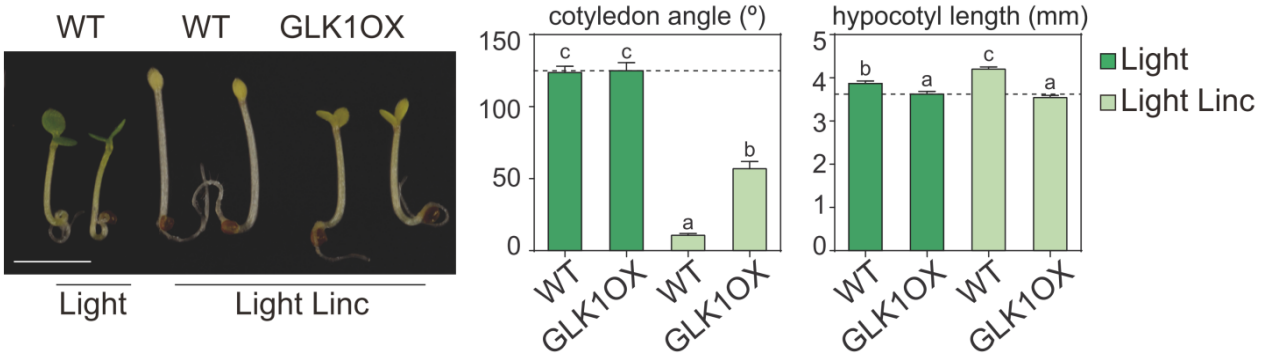


Figure 37. *GLK1OX* is partially resistant to lincomycin. Visual phenotype (left), quantification of cotyledon angle (middle) and hypocotyl length (right) of 3 day-old light-grown WT, and GLK1 overexpressing seedlings (*GLK1OX*) in the absence (dark green) or presence (light green) of lincomycin (Linc). Bar = 2.5 mm. Data are means ± SE of at least 50 seedlings and different letters denote statistically significant differences among means (Tukey-b).

2.9 GLK1 and GLK2 collectively promote photomorphogenesis.

GLK1 belongs to a family of a transcription factors formed by GLK1 and GLK2 (Fitter *et al.*, 2002). So we decided to analyze if these transcription factors act together to induce photomorphogenesis. First, we analyzed the expression of *GLK2* in seedlings grown as detailed in Figure 34. Interestingly, we observed that *GLK2* had a similar expression pattern than *GLK1*: it is a PIF-repressed and light-induced gene, and is strongly repressed in response to lincomycin in WT and *abi4* mutant, but not in *gun1* seedlings (Figure 38). Moreover, *GLK2* has also been described as a PIF target gene (Pfeiffer *et al.*, 2014; Song *et al.*, 2014), suggesting that GLK2 may also act as integrator of dark signals transduced by PIFs and RS signals transduced by GUN1.

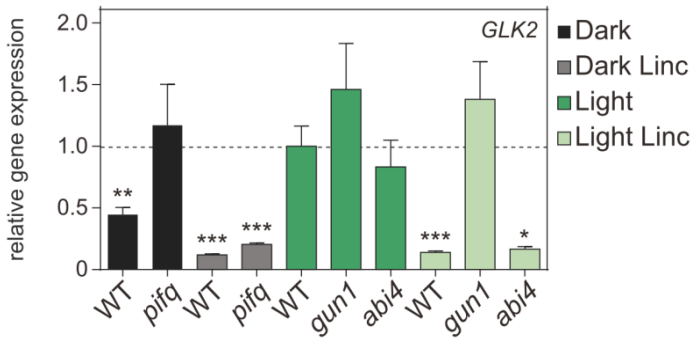


Figure 38. *GLK2* is repressed in the dark and by retrograde signaling through PIFs and GUN1, respectively. Expression of *GLK2* was analyzed by quantitative RT-PCR in 3-day-old seedlings grown in the dark (Dark), dark plus lincomycin (Dark Linc), continuous white light (Light) and continuous light plus lincomycin (Light Linc) seedlings. Values were normalized to *PP2A* and expression levels are shown relative to WT-light set at unity (dashed line). Data are the means ± SE of biological triplicates and asterisks indicate statistically significant differences from light-grown WT seedlings by Student's *t* test.

In addition, we analyzed the expression profile of the previously defined 41 GLK2-upregulated genes that are present in ATH1 array. GLK2-regulated genes were defined in the same transcriptomic analysis as GLK1 (Waters *et al.*, 2009), and the analysis here was performed as in Figure 35 for the GLK1-upregulated genes. Interestingly, GLK2-upregulated genes were globally upregulated in *pifq* dark-grown seedlings to similar levels than those exhibited by WT light-grown seedlings (Figure 39A). Remarkably, GLK2-upregulated genes were also repressed in response to lincomycin, and the expression level were similar to those of WT dark-grown seedlings (Figure 39A). In contrast, gene expression in lincomycin-treated light-grown *gun1* was not statistically significantly different to that of WT-light seedlings because this mutant responds to lincomycin less than WT. Moreover, similarly to GLK1-upregulated genes, we observed an enrichment of PIF-RS

genes among GLK2-upregulated genes (Figure 39B), Altogether, these results suggest that GLK2 and GLK1 may act downstream of the PIFs to collectively promote the expression of a light-induced transcriptional network, which is in turn repressed by retrograde signals transduced by GUN1.

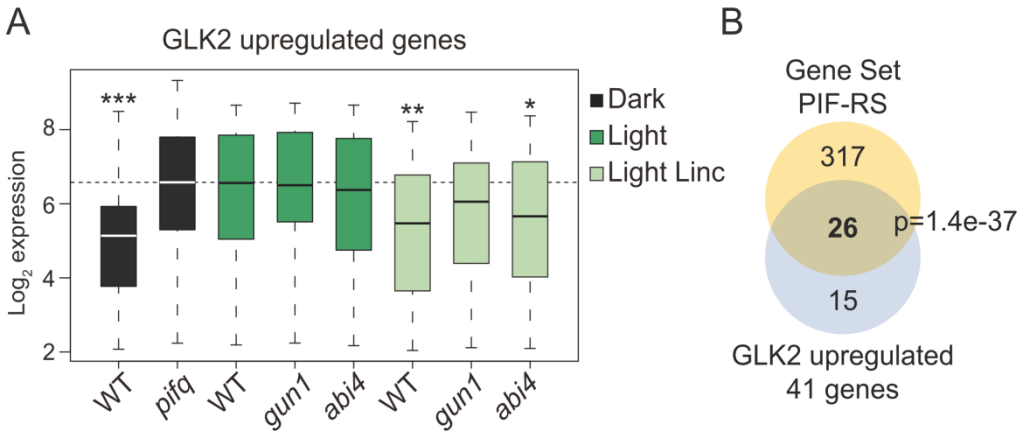


Figure 39. GLK2-upregulated genes are significantly enriched in PIF-RS genes. **(A)** Boxplot representation of Transcript levels of a set of 41 genes previously defined as GLK2-induced (Waters *et al.*, 2009) that have a corresponding probe in the ATH1 array. Data are from microarray experiments that included 2 day-old dark-grown WT and *pifq* (black), and 5 day-old light-grown WT, *gun1* and *abi4* in the absence (dark green) or presence (light green) of lincomycin. Statistically significant differences from WT light by heteroscedastic t test are indicated. Data obtained from GSE5770 and GSE17159 (Koussevitzky *et al.*, 2007; Leivar *et al.*, 2009). **(B)** Venn diagram showing the overlap between the 343 genes in gene set PIF-RS and the 41 genes upregulated by GLK2 (Waters *et al.*, 2009) that have a corresponding probe in the ATH1 array. P-value corresponds to hypergeometric statistical test to assess the significance of the number of PIF-RS genes in the GLK2 upregulated gene set compared to the total population of genes represented on the ATH1 array.

Given that the transcriptional regulation of *GLK2* and GLK2-upregulated genes is also consistent with a possible role of this gene in integrating light and RS signals, we tested the role of GLK2 regulating photomorphogenesis. For this purpose, we first analyzed the phenotype of *glk1*, *glk2* and *glk1glk2* mutants grown for 3 days in continuous white light. Single and double mutants showed a different degree of partial suppression of photomorphogenic development in the light, with more closed cotyledons and longer hypocotyls than WT (Figure 40A). These data suggest that both GLK1 and GLK2 transcription factors act as positive regulators of photomorphogenesis in the light. To assess their participation as intermediates of the phytochrome signaling pathway, we also analyzed the photomorphogenic phenotype of these mutants in continuous red light. We concluded that both GLK1 and GLK2 also act as positive regulators of phytochrome signaling in red light, with GLK2 appearing to play a more prominent role under these conditions than in white light

(Figure 40B). In addition, we did not see any effect of these mutations in skotomorphogenesis (Figure 40C), suggesting that GLKs mainly function in the light.

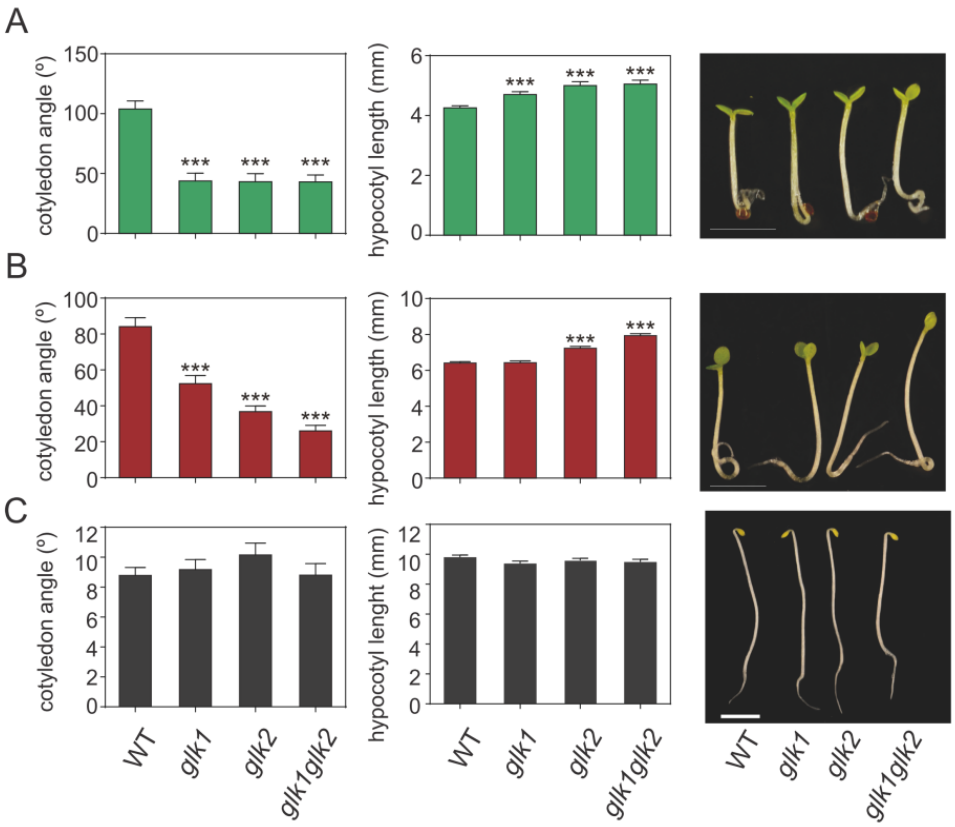


Figure 40. GLK1 and 2 induce photomorphogenesis collectively. Quantification of cotyledon angle (left) and hypocotyl length (middle), and visual phenotype (right), of WT, *glk1*, *glk2* and *glk1glk2* mutants grown for 3 days under continuous white light **(A)**, under continuous red light **(B)** or in the dark **(C)**. Data are means ± SE of at least 35 seedlings and asterisks indicate statistically significant differences from the corresponding WT seedlings by Student's *t* test.

Next, we tested the role of GLK2 in repressing photomorphogenesis downstream of the RS pathway, by analyzing the lincomycin response of *GLK2OX* lines in a similar way than we did for *GLK1OX* (Figure 37). In contrast to *GLK1OX*, we observed that lincomycin strongly repressed the cotyledon aperture of *GLK2OX* seedlings grown for 3 days in the light, suggesting that RS-induced repression of photomorphogenesis is no mediated by GLK2 downregulation. However, we observed that hypocotyls of *GLK2OX* seedlings did not elongate in response to lincomycin, suggesting that GLK2 may mediate this part of the lincomycin effect (Figure 41). Together, these data indicate that, although its expression is strongly inhibited by lincomycin, GLK2 does not seem as important as GLK1 in mediating the RS-induced suppression of the morphological phenotypes associated with photomorphogenesis. However, the transcriptional data showing that GLK2 repressed expression of

PIF-RS subset of genes (Figure 39), which is enriched in chloroplast proteins (Figure 29B), suggested that GLK2 may be important for mediating the RS-induced downregulation of PhANGs.

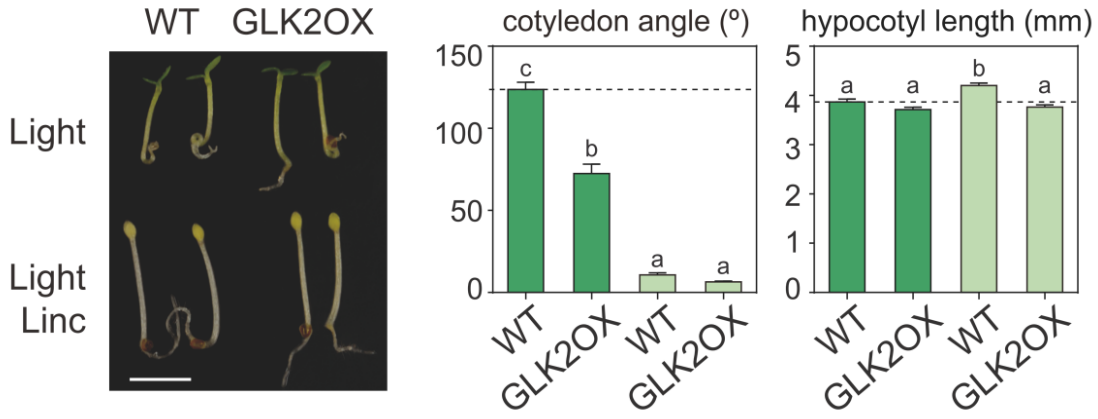


Figure 41. *GLK2* regulation of photomorphogenesis in lincomycin-treated seedlings. Visual phenotype (left), quantification of cotyledon angle (middle) and hypocotyl length (right) of 3 day-old light-grown WT, and *GLK2* overexpressing (GLK2OX) seedlings in the absence (dark green) or presence (light green) of lincomycin (Linc). Bar = 2.5 mm. Data are means ± SE of at least 50 seedlings and different letters denote statistically significant differences among means (Tukey-b).

Because of the incomplete overlap between PIF-RS and GLK1- GLK2 regulated genes, we explore the possibility that other transcription factors regulated photomorphogenesis in addition to GLKs. Comprehensive analysis of our RNAseq data showed that there are 17 transcription factors that similarly to GLKs, are coregulated in the same direction by PIFs and retrograde signals (Table 3). This result makes these transcription factors candidates to also repress the PIF-RS gene set in the darkness and in response to retrograde signals. Among them, the majority have been related to physiological process other than photomorphogenesis, ranging from germination (GATA8, SPATULA (Liu *et al.*, 2005), (Penfield *et al.*, 2005)), plant stress responses (RAV2, WRKY 25 and NF-YB3 (Li *et al.*, 2009; Liu and Howell, 2009; Fu *et al.*, 2014)), carpel development (SPATULA, (Reymond *et al.*, 2012)) to flowering (CIB5 and RAV2 (Liu *et al.*, 2013; Matías-Hernández *et al.*, 2014)). Importantly, we found five transcription factors belonging to the BBX family of plant transcription factors, some of whose members have been previously related to photomorphogenesis (Gangappa and Botto, 2014). The overrepresentation of this family of transcription factors suggested that there may exist an important connection between this family and the repression of seedling deetiolation in the dark and in response to lincomycin, as we demonstrated for BBX16 (discussed in chapter 3).

Table 3: Transcription factors PIF-regulated with a lincomycin reversion percentage bigger than 75. The AGI number (left), the gene name (middle), and their regulation by PIFs and RS is shown for each of the transcription factors whose gene expression is regulated by PIFs, and restored by Lincomycin in *pifq* dark-grown seedlings.

AGI	Name	PIF and RS regulation
AT4G26150	CGA1	Repressed
AT1G25440	BBX15	Repressed
AT1G68840	ATRAV2	Repressed
AT3G02380	CONSTANS-like 2	Repressed
AT1G68520	BBX14	Repressed
AT1G73870	CONSTANS-like 7;BBX16	Repressed
AT5G15850	CONSTANS-like 1, BBX2	Repressed
AT4G14540	NF-YB3	Repressed
AT3G11090	LBD21	Repressed
AT4G36930	SPATULA	Induced
AT2G30250	WRKY25	Induced
AT3G11580	AP2/B3-like transcriptional factor	Induced
AT3G54810	GATA8	Induced
AT1G14600	Homeodomain-like superfamily protei	Induced
AT1G26260	cryptochrome-interacting basic-helix-loop-helix 5	Induced
AT4G25990	CCT motif family protein	Induced
AT5G53420	CCT motif family protein	Induced

Interestingly, among these transcription factors we found CGA1, which is directly repressed by PIFs in the dark (Richter *et al.*, 2010), regulates chloroplast development (Chiang *et al.*, 2012), and acts downstream of gibberellins and auxin hormones to regulate different aspects of plant development (Richter *et al.*, 2013). This suggested that CGA1 may repress photomorphogenesis collectively with GLKs; however, experimental analysis of seedlings constitutively expressing *CGA1* demonstrated that CGA1 was not repressing photomorphogenesis in response toRS (Figure 42).

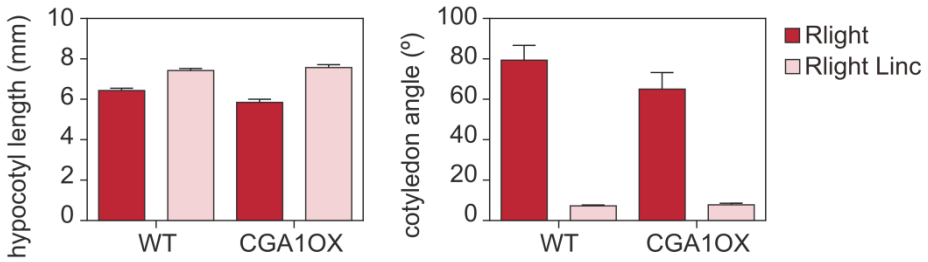


Figure 42. *CGA1OX* seedlings grown in presence of lincomycin. Quantification of hypocotyl length (left) and cotyledon angle (right) of WT and *CGA1OX* overexpressing line grown for 3 days in red light in the absence (Rlight) or presence (Rlight Linc) of lincomycin (left). Data are means ± SE of at least 25 seedlings.

2.10 Excess light represses photomorphogenesis.

To better understand the physiological implications of the interplay between PIF and retrograde signals, and because a major function of the PIFs is to drive the rapid developmental switch of early deetiolation, we next examined the possibility that the identified interplay occurs during the dark-to-light transition. For this, we studied the impact of lincomycin upon exposure of 2 day-old dark-grown seedlings to light. Remarkably, the fast and robust hook opening and cotyledon separation exhibited by dark-grown seedlings transferred to light was nearly completely blocked in the presence of lincomycin (Figure 43), suggesting that the antagonizing action of RS on light/PIF signaling can occur during early deetiolation.

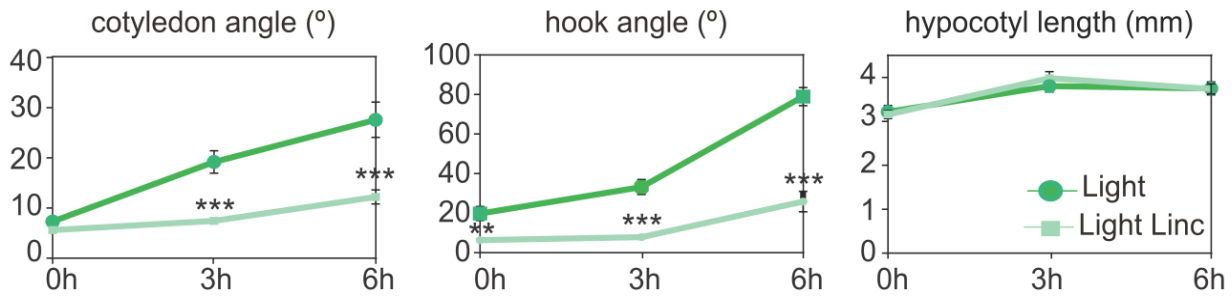


Figure 43. Lincomycin inhibits early deetiolation during the transition of dark-grown seedlings to light. Quantification of cotyledon angle (left), hook angle (middle) and hypocotyl length (right) of 2-day-old dark-grown WT seedlings transferred to white light for the indicated times grown in the absence (dark green) or presence (light green) of lincomycin. Data are means ± SE of at least 25 seedlings and asterisks indicate statistically significant differences between mean values by Student's t test.

Moreover, given our results that lincomycin acts to repress early seedling deetiolation (Figure 43), together with the fact that RS is activated in natural environments when chloroplast perceive high light conditions (Pfannschmidt and Yang, 2012), we hypothesized that seedlings emerging from soil surface that are exposed to potentially damaging excess light would repress early deetiolation response through RS. To test this possibility, we examined the response to excess light of dark-grown seedlings. First, we measured the expression of the well-established light-induced RS-repressed genes *LHCB1.4* and *LHCB2.2* in seedlings that were transferred from darkness to high light (HL) conditions (Figure 44). Data showed that the rapid light-induced accumulation of these transcripts upon transfer of dark-grown WT seedlings to light (L) was significantly lower in high light. In contrast, *gun1* mutant seedlings accumulated similar amount of *LHCB* transcripts in both L and HL (Figure 44). This result indicate that our HL conditions were inductive of GUN1-mediated RS, in agreement with previous reports (Kleine *et al.*, 2007).

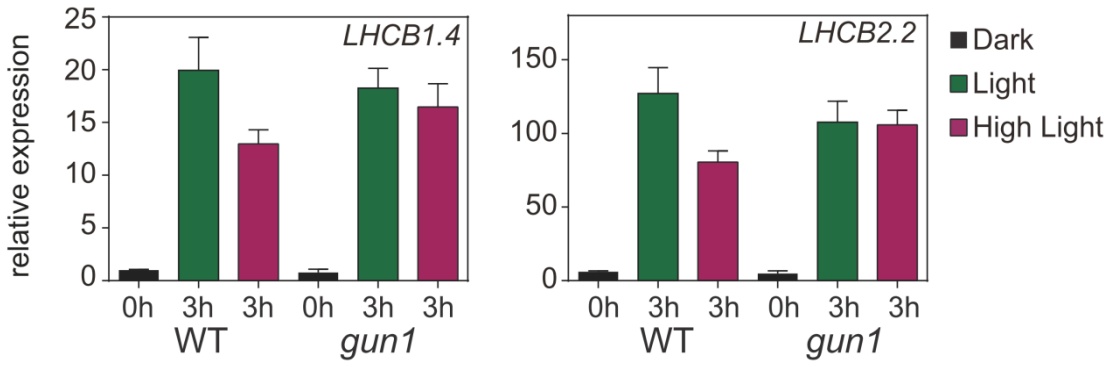


Figure 44. High light conditions induce GUN1-mediated retrograde signaling. Transcript levels of *LHCB1.4* (left) and *LHCB2.2* (right) were analyzed by quantitative qRT-PCR in 2-day-old dark-grown WT and *gun1* transferred for 3 h to white light (L) (green) or high light (HL) (purple). Controls were harvested in the dark at time 0 (black). Values were normalized to *PP2A* and expression levels are expressed relative to WT dark set at one. Data are the means ± SE of biological triplicates.

We next examined the seedlings phenotypically under these conditions. The data showed that hook unfolding and cotyledon separation were significantly slower in WT seedlings exposed to 3 and 6 h of HL compared to L (Figure 45). In contrast, *gun1* mutant seedlings were insensitive to the HL, and deetiolated at the same rate in both L and HL (Figure 45). Together, these results indicate that GUN1-mediated RS activated by excess light during early seedling deetiolation can antagonize the light induction of the photomorphogenic program.

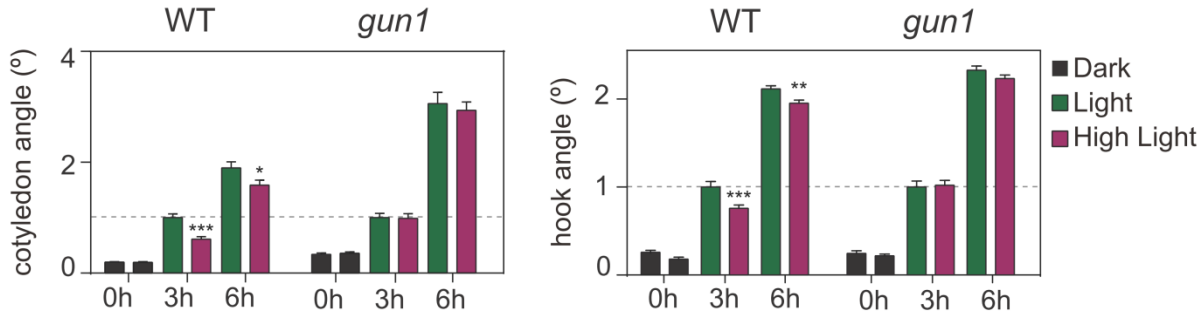


Figure 45. GUN1-mediated Retrograde Signaling is induced by high light and inhibits seedling deetiolation. Cotyledon separation (left) and hook unfolding (right) in dark-grown WT and *gun1* mutant seedlings after 3 and 6 h of white light (green) or High Light (purple) treatments. Values for each genotype are expressed relative to the corresponding value in L at 3h set at one. Data are means ± SE of at least 60 seedlings and asterisks indicate statistically significant differences from light seedlings by Student's *t* test.

2.11 *High light repression of GLK1 expression is mediated by RS and not by PIF activity.*

In addition, because of the important role of GLK1 as downstream integrator of light and RS signals transduced by the PIFs and by GUN1 (see above), we next studied whether GLK1 also mediates the high-light suppression of photomorphogenesis during early deetiolation. First, we analyzed *GLK1* expression in our RS-inductive HL conditions during early deetiolation. Interestingly, we observed that the rapid L induction of *GLK1* expression was significantly reduced in HL, an effect that was not observed in *gun1* mutants where *GLK1* expression was similar in L and HL (Figure 46A), thus suggesting that HL can antagonize the light induction of *GLK1* expression at least in part through GUN1-mediated RS. To analyze the global expression profile of the GLK1-regulated transcriptional network in response to HL, we next studied the expression of the subset of GLK1-upregulated genes using available microarray data of seedlings exposed to high light for 3 hours (Kleine *et al.*, 2007). Consistent with the HL-repression of GLK1 expression (Figure 46A), we observed that HL globally represses the expression of GLK1-induced genes, a behavior that was also found for the genes in the 'Gene Set PIF-RS' and the genes down-regulated by RS (Figure 46B).

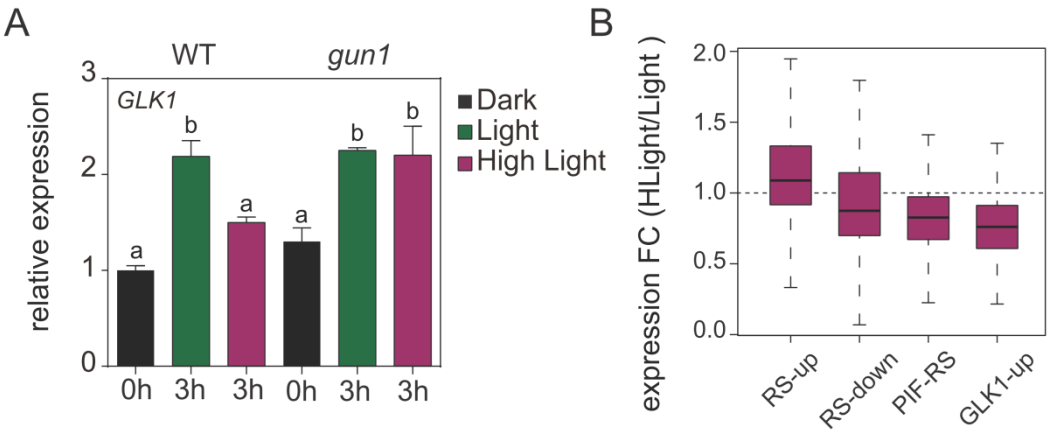


Figure 46. High light represses GLK1 expression and transcriptional network (**A**) Transcript levels of *GLK1* were analyzed by quantitative qRT-PCR in 2-day-old dark-grown WT and *gun1* transferred for 3 h to white light (L) (green) or high light (HL) (purple). Values were normalized to *PP2A* and expression levels are expressed relative to WT dark set at one. Data are the means ± SE of biological triplicates and different letters denote statistically significant differences among means (Tukey-b). (**B**) Boxplot representation of mean fold-change (FC) expression of WT seedlings grown in HL and L (HLight/ Light) for the following Gene Sets: Retrograde Signaling (RS) upregulated, RS downregulated genes (Woodson *et al.*, 2013), gene set PIF-RS (Figure 26), and GLK1 upregulated (Waters *et al.*, 2009). Data obtained from GSE7743 (Kleine *et al.*, 2007).

Because *GLK1* is a PIF-repressed gene, one simple explanation of the HL-repression of *GLK1* is that PIFs reaccumulate under these conditions. To test this possibility, we studied PIF3 protein levels in our HL conditions in WT and *gun1* mutant seedlings (Figure 47). Because we did not detect such an increase in PIF3 levels, which remained below detection in WT and *gun1* in both L and HL (Figure 47), our findings agree with the notion that *GLK1* expression under HL is repressed in a PIF-independent fashion by GUN1-mediated retrograde signaling.

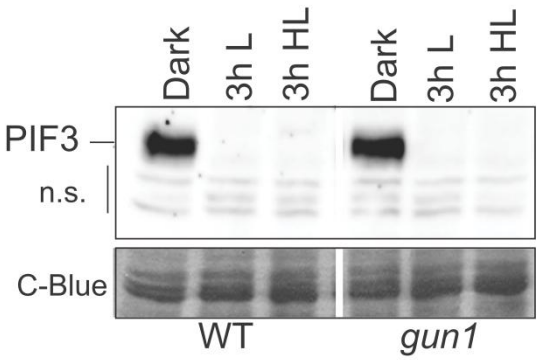


Figure 47. PIF3 is not accumulated under High Light conditions. Immunoblot of protein extracts of 2-day-old dark-grown (Dark) WT and *gun1* transferred for 3 h to white light (L) or high light (HL). Comassie staining was used as a loading control (bottom). n.s., non-specific cross-reacting bands.

Given the observed RS-induced repression of *GLK1* expression in response to HL, together with the fact that *GLK1* transcriptional repression is needed to suppress deetiolation in seedlings treated with lincomycin (Figure 37), we predicted that manipulation of *GLK1* levels in *GLK1* overexpressing lines would cause an insensitive phenotype to HL. To explore this possibility, we analyzed seedling deetiolation in *GLK1OX* seedlings transferred from darkness to high light. Interestingly, we found that *GLK1OX* seedlings deetiolated similarly both in L and in HL (Figure 48), especially regarding the cotyledon angle phenotype, thus suggesting that *GLK1* overexpression causes insensitivity to HL compared to WT seedlings (shown in Figure 45). This result provides a further support to our hypothesis that *GLK1* repression is required for the suppression of photomorphogenesis mediated by chloroplast-derived retrograde signals.

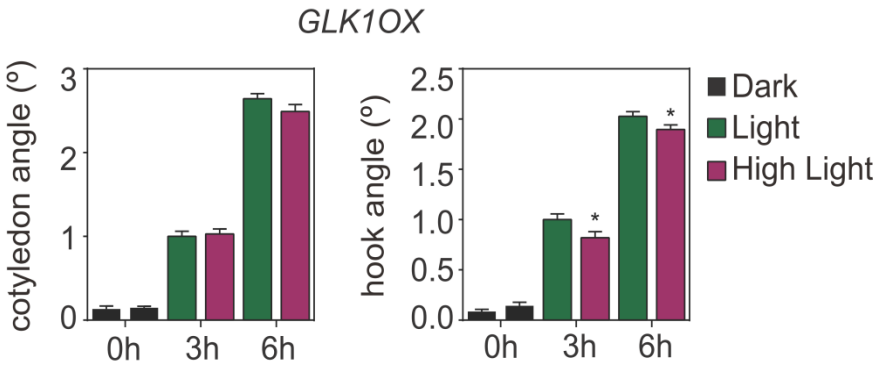


Figure 48. *GLK1-OX* seedlings are largely insensitive to HL. Cotyledon separation (left) and hook unfolding (right) in dark-grown *GLK1* overexpressing (GLK1-OX) seedlings after 3 and 6 h of white light (L) (green) or high light (purple) treatments. Values are expressed relative to the corresponding value in L at 3h set at one. Data are means ± SE of at least 40 seedlings and asterisks indicate statistically significant differences from light seedlings at each time point by Student's *t* test.

3. BBX16 implements the morphological aspects of seedling photomorphogenesis downstream of GLKs.

3.1 GLKs regulate a subset of members of the BBX transcription factor family.

Given that our results demonstrate that PIFs and retrograde signals regulate photomorphogenesis through the transcriptional regulation of GLKs, and because the GLK-regulated genes in the light were defined previously (Waters *et al.*, 2009), we focused on the GLK-regulated genes under light conditions (Figure 35, 39) with the purpose of finding some candidate genes that could be responsible for regulating morphological features of photomorphogenesis downstream of GLKs. Most of GLK1 and GLK2-upregulated genes are associated to the photosynthetic apparatus, which correlates with previous reports showing that GLKs induce photosynthetic apparatus formation by directly inducing the expression of these genes (Waters *et al.*, 2009). Accordingly, in our conditions, light-grown *glk1glk2* double mutants show a pale phenotype and express reduced levels of *LHCB.1.4* compared to WT seedlings (Figure 49) (Fitter *et al.*, 2002). Because of this result, we reasoned that the transcriptional regulation of chloroplast proteins by GLKs could explain mainly the formation of photosynthetic apparatus and chloroplast biogenesis after light exposure. However, morphological changes that occur after light exposure must be dependent on other GLK-regulated genes that have not yet been identified.

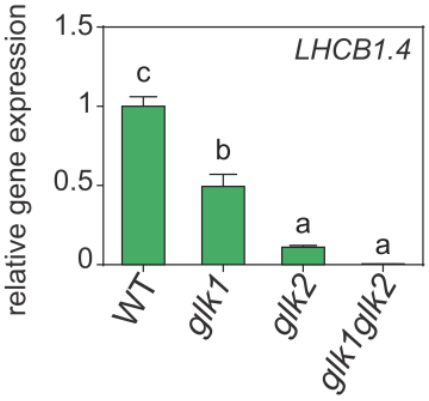


Figure 49. GLKs induce *LHCB1.4* expression under light conditions. Expression of *LHCB1.4* analyzed by quantitative RT-PCR in 3-day old light-grown WT, *glk1*, *glk2* and *glk1glk2* seedlings. Expression levels relative to wild type (WT) are shown. Data are the means ± SE of technical triplicates and different letters denote statistically significant differences among means (Tukey-b).

Significantly, we observed an enrichment of genes encoding for BBX transcription factors among the GLK-upregulated genes (p-value: 2.46 e-05), since we identified four of the described 32 BBX family members in the list of the 119 genes reported as GLK1- or GLK2-upregulated. Moreover, three of the four BBX were members of the subclass III, which is composed by four members (Khanna *et al.*, 2009).

Given that *BBX*s were among the few genes that were GLK-upregulated and not coding for chloroplast proteins, we decided to study their role as possible downstream effectors of GLK to regulate cotyledon aperture and hypocotyl inhibition in response to light. We focused on class III of *BBX* family because of their overrepresentation among GLK-upregulated genes, and we first determined their expression in *glk1*, *glk2* and *glk1glk2* mutants grown for 3 days in the light to further confirm their regulation by GLKs. Three of them, *BBX14*, *BBX15* and *BBX16* were strongly downregulated in *glk1glk2* (Figure 50). Regarding *BBX17*, it was also misregulated in *glk2* and *glk1glk2* mutants, although the effect was more moderate than in the other three members (Figure 50). These results were consistent with previous microarray data (Waters *et al.*, 2009), demonstrating that GLK1 and GLK2 together regulate the expression of class III *BBX*s.

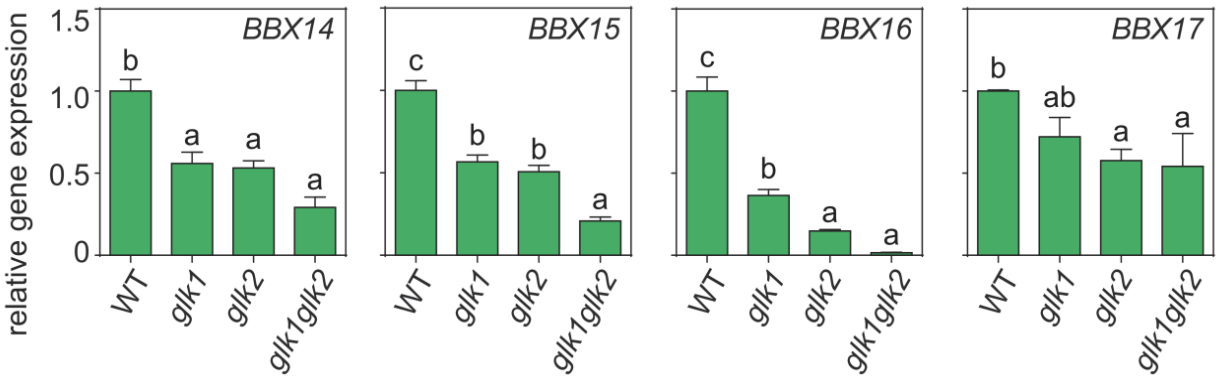


Figure 50. GLKs regulates *BBX* subclass III family of transcription factors under light conditions. Expression of *BBX14*, *BBX15*, *BBX16* and *BBX17* analyzed by quantitative RT-PCR in 3-day old light-grown WT, glk1, glk2 and glk1glk2 seedlings. Expression levels relative to wild type (WT) are shown. Data are the means ± SE of technical triplicates and different letters denote statistically significant differences among means (Tukey-b).

3.2 BBX16 regulates photomorphogenesis.

Considering the above results, we studied whether *BBX* missregulation in *glk* mutants could be responsible for the phenotypic alterations of *glk* mutants in response to light (Figure 40, 49). To study the role of BBX subclass III in regulating photomorphogenesis, we focused on BBX16 transcription factor because it was the most deregulated in *glk* mutants (Figure 50). For this, we

obtained a *bbx16* T-DNA insertion mutant line and two lines overexpressing *BBX16* (OX1 and OX2) (Wang *et al.*, 2013), and quantified cotyledon angle, hypocotyl length and cotyledon area in 3 day-old light-grown seedlings in comparison to their respective WT (Col4). Overexpression lines had shorter hypocotyls and more open and expanded cotyledons than WT seedlings (Figure 51A), strongly suggesting that BBX16 induces photomorphogenesis. In contrast, although the deficiency of *BBX16* in the *bbx16* mutant led to a reduced cotyledon area, the hypocotyls of these mutants were shorter than the WT, and the cotyledon aperture remained unchanged (Figure 51A). The apparent contradictory hypocotyl phenotype among mutant and overexpression lines made it difficult to conclude whether the role of BBX16 in regulating photomorphogenesis is positive or negative. In order to have additional evidence about the function of BBX16, we quantified the deetiolation phenotypes during the early dark to light transition. For this, we performed a time course experiment of 2 day-old dark grown WT and *bbx16* mutants exposed to 6 hours of light. These experiments showed that *bbx16* mutants display a significant reduction in cotyledon aperture compared to WT six hours after light exposure (Figure 51B), thus strongly suggesting that BBX16 induces early photomorphogenesis, at least in the apical part of the seedling.

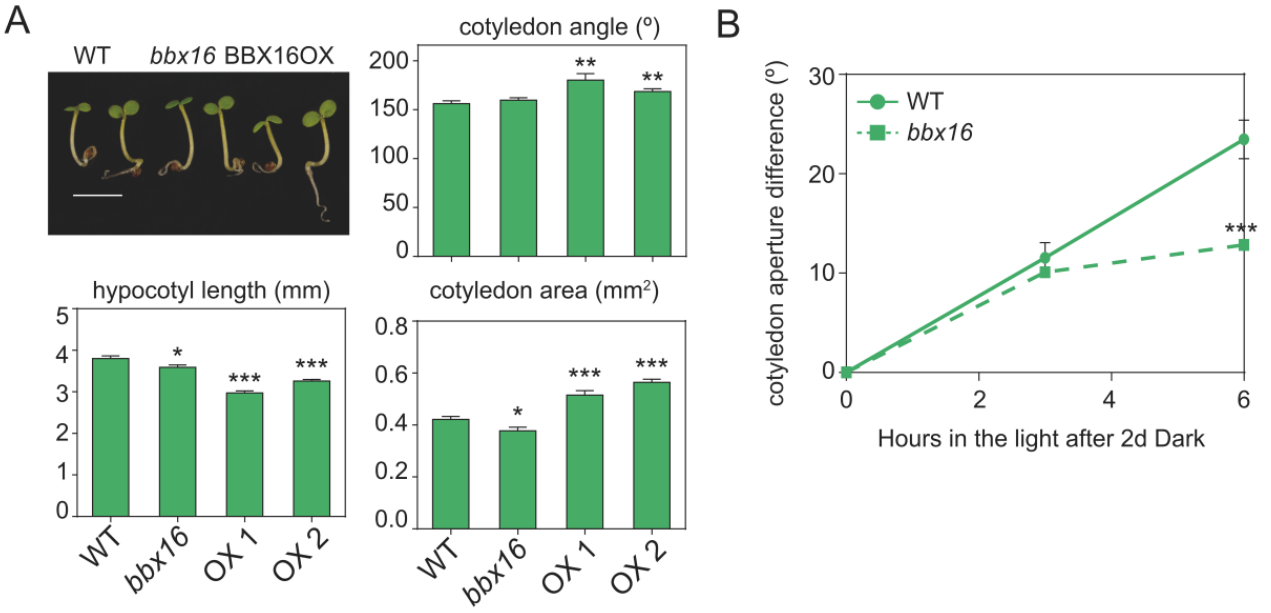


Figure 51. BBX16 induces photomorphogenesis during early seedling development. (A) Visual phenotypes of 3 day-old light-grown WT, *bbx16*, and *BBX16OX* seedlings (top left). Bar = 2.5 mm. Quantification of cotyledon angle (top right), hypocotyl length (bottom left) and cotyledon area (bottom right). Data are means ± SE of at least 35 seedlings. **(B)** Quantification of cotyledon aperture of WT and *bbx16* seedlings grown for 2days in the dark and exposed to 3 or 6 hours of light. Data represent the difference in cotyledon aperture at each time point compared to 0h for seedling of each genotype. Data are means ± SE of at least 45 seedlings. For (A) and (B) asterisks indicate statistically significant differences from WT seedlings by Student's *t* test.

Overall, *BBX16* downregulation in light-grown *glk* mutants (Figure 48), together with the altered photomorphogenic phenotype of *BBX16* overexpressing and mutant lines (Figure 49), suggest that GLKs induce photomorphogenesis after light exposure through the transcriptional induction of *BBX16,* and probably other *BBX* genes of the same family.. To confirm this role of BBX16 downtream of GLKs, ChiP-qPCR analysis of GLK binding to the *BBX16* promoter region is necessary.

3.3 BBX16 represses photomorphogenesis in response to chloroplast dysfunction.

Considering the postulated role of BBX16 in promoting photomorphogenic development under light conditions, we next studied whether BBX16 is also involved in the retrograde signaling pathway that represses photomorphogenesis when chloroplast functionality is compromised.

First, we analyzed the expression pattern of *BBX16* in respone to lincomycin in *GLK1* overexpressing seedlings (*GLK1OX),* which have an attenuated response to lincomycin and are able to partially deetiolate when chloroplasts are damaged (Figure 37). Moreover, this analysis was performed in *GLK2OX* lines, which do not have a prominent role in regulating morphological photomorphogenesis in response to lincomycin (Figure 41). We observed that *BBX16* is strongly upregulated in *GLK1OX* lines grown in the light, an effect not observed in *GLK2OX* lines. These data are consistent with the proposed regulation of *BBX16* by GLK1, and suggest that missregulation of this gene in *glk2* mutants (Figure 50) could depend on other factors. Interestingly, *BBX16* was strongly repressed in response to lincomycin in WT and *GLK2OX*, which is concomitant to a decrease in *GLK1* expression in WT (Figure 34), but this effect was not observed in *GLK1OX* lincomycin-light grown seedlings (Figure 52). Together, these data provide a strong support to the notion that downregulation of *BBX16* by lincomycin is mediated by GLK1, and suggest that *BBX16* downregulation may drive the lincomycin-induced repression of photomorphogenic development,.

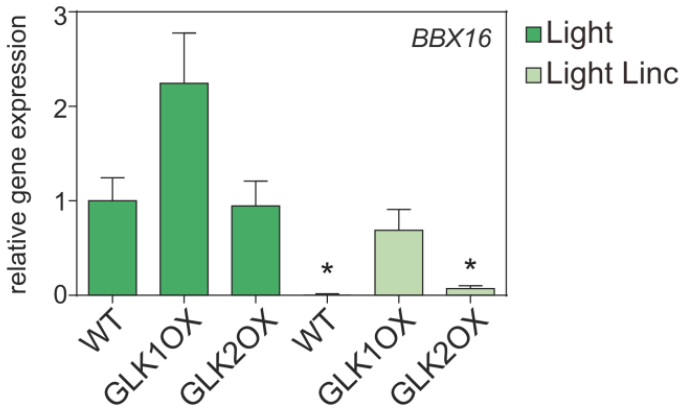


Figure 52. GLK1 represses *BBX16* expression in response to lincomycin. Expression of *BBX16* was analyzed by quantitative RT-PCR in WT, *GLK1OX* and *GLK2OX* seedlings grown for 3 days in white light in the absence (Light) or presence (Light Linc) of lincomycin. Expression levels relative to wild type (WT) light are shown. Data are the means ± SE of biological triplicates and asterisks indicate statistically significant differences from WT light-grown seedlings by Student's *t* test.

To test the postulated role of GLK1-mediated *BBX16* downregulation in repressing photomorphogenesis in response to lincomycin, we grew *BBX16OX* lines for three days in plates containing lincomycin under light conditions. Strikingly, *BBX16* overexpressing lines were insensitive to lincomycin and were able to deetiolate, showing a cotyledon aperture that was almost the same than WT seedlings without lincomycin, and a marginal increase in hypocotyl elongation (Figure 53). As result of this analysis we concluded that *BBX16* downregulation is necessary to the RS-mediated repression of phenotypic photomorphogenesis.

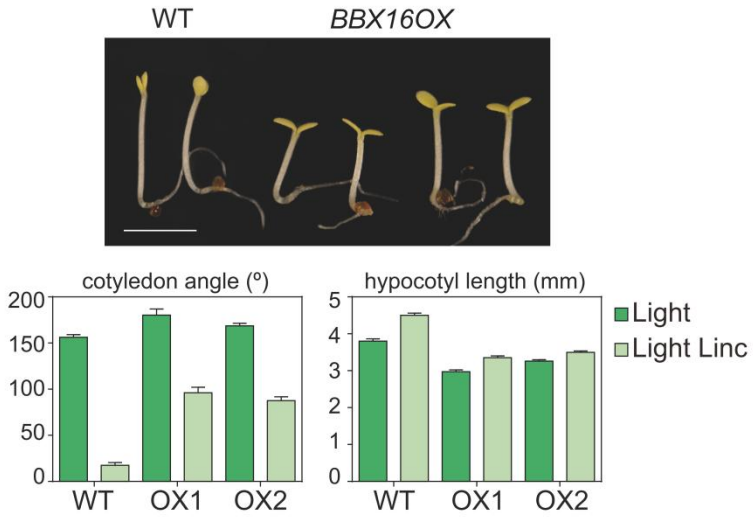


Figure 53. *BBX16* downregulation is necessary to repress photomorphogenesis in response to lincomycin. Visual phenotypes of WT and two *BBX16* overexpressing lines (OX1, OX2) grown for 3 days in white light in the absence

(Light) or presence (Light Linc) of lincomycin (top). Bar = 2.5 mm. Quantification of cotyledon angle (bottom left) and hypocotyl length (bottom right). Data are means ± SE of at least 40 seedlings.

3.4 BBX16 does not regulate expression of photosynthetic genes.

As *LHCB* genes are molecular markers of retrograde signaling activity, we next studied *LCHB1.4* and *LHCB.2.2* expression in 3 day-old light-grown WT and *BBX16OX* seedlings in the presence or absence of lincomycin. Importantly, although *BBX16* overexpressing lines are insensitive to lincomycin at the morphological level (Figure 53), *LHCB.1.4* and *LHCB.2.2* expression was downregulated in *BBX16OX* lines to the same extent as in WT (Figure 54). This result suggest that BBX16 does not mediate the repression of Photosynthetic Associated Nuclear Genes (PhANGs), which is consistent with chip-qPCR data showing that GLK1 directly regulates expression of genes that codify for photosynthetic apparatus proteins such as *LHCB.2.2* (Waters *et al.*, 2009). Hence, this experiment supports the notion that GLK1 branches in a downstream of transcriptional network to separately regulate chloroplast formation (through the transcriptional regulation of PhANGs), and morphological features of photomorphogenic development (through the transcriptional regulation of *BBX16).*

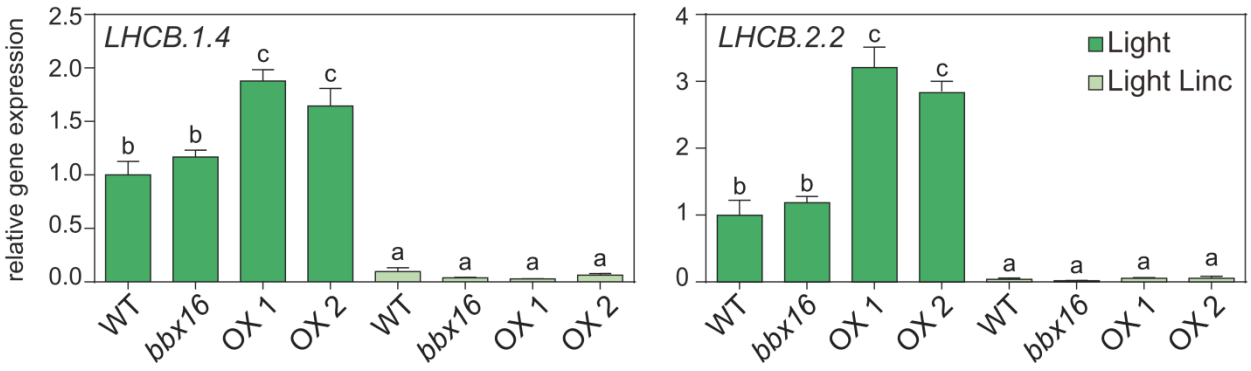


Figure 54. BBX16 does not mediate the repression of *LHCB* genes in response to lincomycin. Expression of *LHCB1.4* (left) and *LHCB2.2* (right) was analyzed by quantitative RT-PCR in WT, *bbx16* and *BBX16OX* seedlings grown for 3 days in white light in the absence (Light) or presence (Light Linc) of lincomycin. Expression levels relative to wild type (WT) light are shown. Data are the means ± SE of biological triplicates and different letters denote statistically significant differences among means (Tukey-b).

4. CDF5 links the clock and light-signaling to regulate growth.

4.1 Cicling Dof Factors (CDF) repress photomorphogenesis.

Because of the interesting phenotype of *SOP90*, a *pifq* suppressor mutant with longer hypocotyl and closed cotyledons (Table 1), together with the confirmation that *SOP90* overexpresses *CDF2* (Figure 55A), we decided to study the interplay between CDFs and PIFs in regulating photomorphogenesis. CDFs (Cicling Dof Factor) are a subgroup of DOF transcription factors including five members (CDF1-5) that repress flowering in short days (Fornara *et al.*, 2009). First, to validate the role of *SOP90* in regulating photomorphogenesis, we propagated individual T2 plants to obtain the T3 generation, and then performed phenotypic studies with homozygous *SOP90* lines (CDF2OX*pifq* lines) and *pifq* siblings (CDF2WT*pifq*). *CDF2* overexpressing seedlings had longer hypocotyls and closed cotyledons compared to *pifq* seedlings grown for three days in the dark, indicating that the overexpression of *CDF2* in *SOP90* represses photomorphogenesis in the *pifq* genetic background (Figure 55B, 55C).

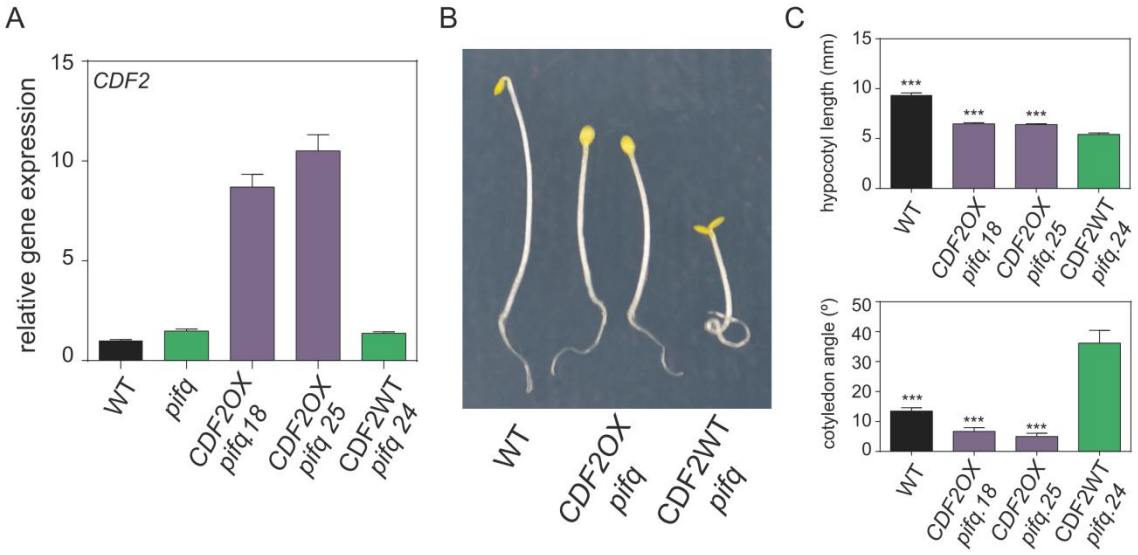


Figure 55. *CDF2* overexpression suppresses *pifq* photomorphogenic phenotype in the dark. (A) Transcript levels of *CDF2* analyzed by quantitative qRT-PCR in WT, *pifqCDF2* overexpressing lines (CDF2OX*pifq*) and *pifq* siblings (CDF2WT*pifq*) grown for three days in the dark. Expression levels relative to WT dark are shown. Data are the means ± SE of technical triplicates **(B)** Visual phenotypes of WT, CDF2OX*pifq* and CDF2WT*pifq* grown for three days in the dark. **(C)** Quantification of hypocotyl length (top) and cotyledon aperture (bottom) of seedlings grown as in (B). Data are means ± SE of at least 20 seedlings and asterisks indicate statistically significant differences from *pifq* siblings by Student's *t* test.

Next, we analyzed the expression of the five described CDFs (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009) in WT and *pifq* seedlings grown in the dark by quantitative RT-PCR. Interestingly, we found that *CDF2* and *CDF3* are PIF-repressed genes, whereas *CDF5* is a PIF-induced gene (Figure 56). *CDF1* levels were below detection range both in WT and *pifq* seedlings, indicating that this gene is not expressed in the dark, whereas CDF4 was not missregulated in *pifq* seedlings compared to WT (Figure 56). *CDF2* showed an intriguing pattern of regulation, because the qPCR results show that the photomorphogenic *pifq* mutants display elevated levels of endogenous *CDF2* gene. However, this upregulation may not be the cause of the photomorphogenic phenotype of *pifq*, since ectopic overexpression of *CDF2* in *pifq* leads to a suppression of the constitutive photomorphogenic phenotype of *pifq* (Figure 55).

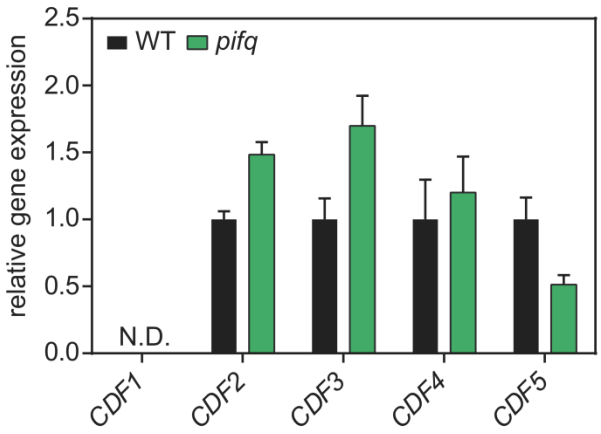


Figure 56. *CDF2, 3* and *5* are regulated by the PIFs in the dark. Transcript levels of *CDF1, 2, 3, 4* and *5* analyzed by quantitative qRT-PCR in 3-day-old dark-grown WT and *pifq* seedlings. Expression levels of each *CDF* are relative to the WT dark sample. N.D. (not detected). Data are the means ± SE of technical triplicates.

4.1.1CDF5 plays a more prominent role among CDFs in regulating photomorphogenesis under continuous red light and under diurnal conditions.

Given the *CDF* expression pattern in *pifq* dark-grown seedlings (Figure 56), we decided to investigate the role of CDF2, CDF3 and CDF5 in regulating seedling development under dark and light conditions, where PIFs have been described to be key regulators (Leivar and Monte, 2014). For this purpose, we studied the photomorphogenic phenotype of *cdf2, cdf3* and *cdf5* single and double mutants grown for 3 days in the dark, red light, short days and long days (Figure 57). Despite the interesting *CDF* expression pattern in *pifq* dark-grown seedlings, and the phenotype observed in *CDF2OX pifq* lines in the dark (Figure 55), *cdf2, cdf3* and *cdf5* single mutants, and all combinations of double mutants did not show any apparent phenotype in darkness, suggesting that CDF2, CDF3 and CDF5 do not play a major role in regulating skotomorphogenesis in the WT

genetic background. These contradictory observations, at least for *CDF2*, may be due to the constitutive ectopic expression of the gene in all cells of the *CDF2OXpifq* lines driven by the 35S promoter. In contrast, under red light or in diurnal conditions (light/night cycles), *cdf* mutant combinations displayed different degrees of expanded cotyledons and/or shorter hypocotyls compared to WT seedlings (Figure 57). Among the different combinations of *cdf* mutations, we observed that those including *cdf5* mutation had the strongest disruption of photormorphogenic development. These results indicate that under these light conditions, CDFs act as repressors of photomorphogenesis, with CDF5 playing a more prominent role, and with CDF3 and CDF2 respectively playing an intermediate and a marginal role.

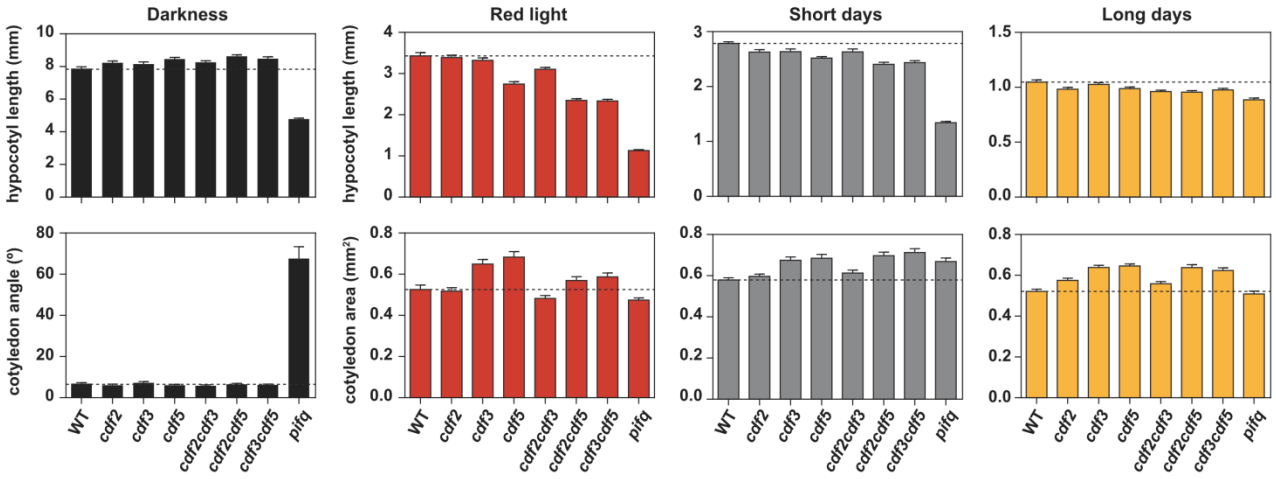


Figure 57. Role of CDFs in regulating photomorphogenesis. Quantification of hypocotyl length (top), cotyledon aperture (left bottom) or cotyledon area (three right bottom) of WT, *pifq* and *cdf* single and double mutants grown for three days in the dark (black), in red light (red), in short days (8h light:16h dark, grey) or long days (16h light:8h dark, yellow). Data are means ± SE of at least 25 seedlings.

Moreover, a time course experiment of *cdf* single mutants over seven days of growth in short day conditions showed that CDF function in repressing photomorphogenesis is maintained until the emergence of true leaves (which approximately occurs after seven days in short days) (Figure 58). In this time course experiment, we confirmed the short hypocotyl phenotypes of *cdf3* and *cdf5* mutants, whereas the effect of the *cdf2* mutation was again marginal.

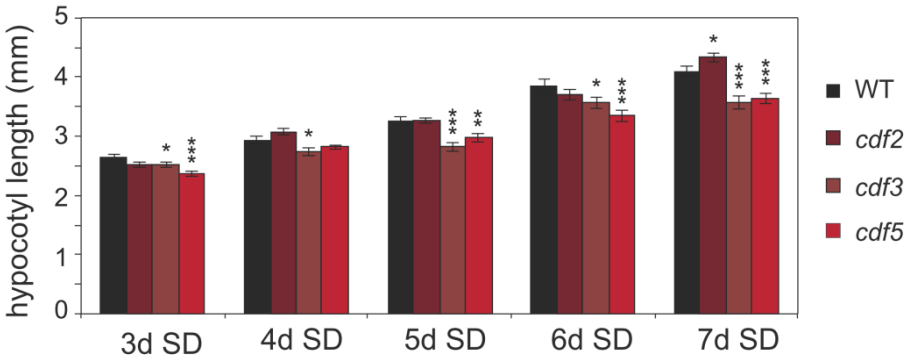


Figure 58. Role of CDFs in promoting hypocotyl growth in short days. Quantification of hypocotyl length of WT and *cdf1*, *cdf2 and cdf3* mutants grown from three to seven days in short days (SD). Data are means ± SE of at least 20 seedlings and asterisks indicate statistically significant differences from WT in each time point by Student's *t* test.

4.2 CDF5 overexpression causes repression of photomorphogenesis under constant red light and under diurnal conditions.

Because of the interesting phenotype of *cdf5* mutants in all tested conditions, we decided to study in more detail its role in repressing photomorphogenesis. For this, we generated *CDF5* overexpressing plants (*cdf5CDF5OX*) in the *cdf5* mutant background, in which *CDF5* is expressed under the control of the strong and constitutive 35S promoter. In red light, *cdf5CDF5OX* lines were longer and had smaller cotyledons than WT seedlings (Figure 59), further confirming the role of CDF5 as repressor of photomorphogenesis.

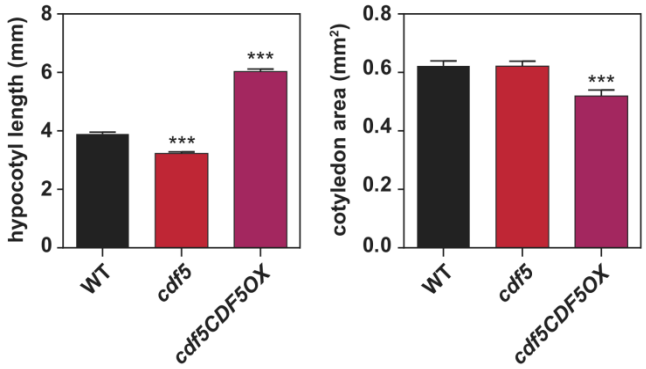


Figure 59. *CDF5* overexpression causes repression of photomorphogenesis in red light. Quantification of hypocotyl length (left) and cotyledon area (right) of three day-old red light grown WT, *cdf5* and *cdf5CDF5OX* seedlings. Bar=2,5mm. Data are means ± SE of at least 25 seedlings and asterisks indicate statistically significant differences from WT by Student's *t* test.

In addition, we also analyzed the phenotype of the overexpressing lines under diurnal conditions. Under short days, we also observed that *CDF5* overexpression causes repression of photomorphogenesis, by showing longer hypocotyls than WT seedlings and reduced cotyledon area

(Figure 60A, 60B). In contrast, *CDF5* overexpression had only a subtle but still significant effect in long day-grown seedlings (Figure 60A, 60B), which is consistent with the observed minor effect of the *cdf* mutations in long days (Figure 57). These data are also in agreement with the fact that CDF protein levels are regulated in a seasonal manner, accumulating less in long days than in SD (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009)..

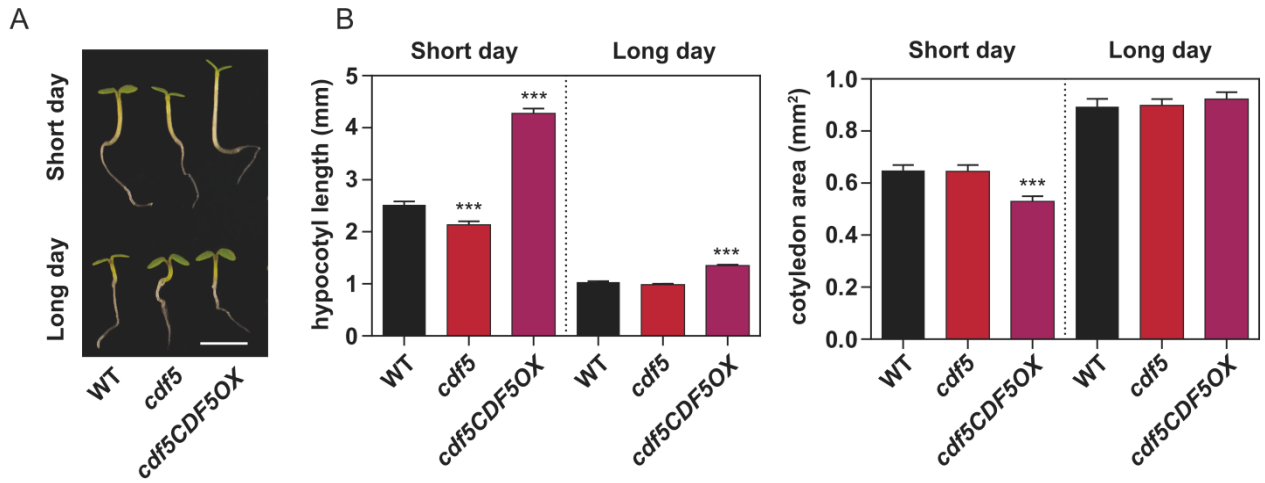


Figure 60. *CDF5* overexpressing lines repress photomorphogenesis in short days. (A) Visual phenotypes of WT, *cdf5* and *cdf5CDF5OX* three day-old SD and LD-grown seedlings. Bar=2,5mm. **(B)** Quantification of hypocotyl length (middle) and cotyledon area (right) of seedlings grown as in (A). Data are means ± SE of at least 20 seedlings and asterisks indicate statistically significant differences from WT by Student's *t* test.

4.3 CDF5 expression is induced at the end of the night to promote growth.

Given the role of CDF5 in promoting growth under diurnal conditions, and because it has been demonstrated that seedling growth in short days is rhythmic with a peak of growth at dawn (Nozue *et al.*, 2007), we next asked whether CDF5 was involved in this rhythmic regulation of hypocotyl length under diurnal conditions. For this purpose, we measured hypocotyl elongation of WT and *cdf5* seedlings during the third day of growth in short days, and calculated the growth that occurred during the 8h light (between ZT0 and ZT8) or 16h night (between ZT8 and ZT24) periods. As control, we grew WT seedlings in free running conditions (2 days in short days plus 1 day in continuous light). In agreement with previous work performed in our laboratory (Soy *et al.*, 2014), growth associated to the night hours (ZT8-ZT24) was strongly inhibited in WT seedlings grown under free running conditions compared to those grown in short days (Figure 61A), confirming that the long night of short-days are required to promote growth. Interestingly, the growth pattern of *cdf5* mutants in short days was similar to that of seedlings grown in free running conditions, and

only grew during day hours (Figure 61A). This result indicates that CDF5 is necessary to induce growth in short days during the dark hours of the day.

Next, to check whether the transcriptional regulation of *CDF5* in diurnal conditions correlates with the growth pattern of seedlings grown in short days, we performed a time course experiment to measure *CDF5* expression in WT seedlings grown in the same conditions as in Figure 61A. Interestingly, this experiment showed that *CDF5* gene expression is rhythmic and peaks at the end of the night in SDs. In contrast, although under free running conditions *CDF5* expression was still oscillating, the amplitude of the peaks was strongly reduced (Figure 61B). These data show that *CDF5* accumulation in short days is strongly dependent on the dark hours, coinciding with the CDF5-dependent induction of growth (Figure 61A).

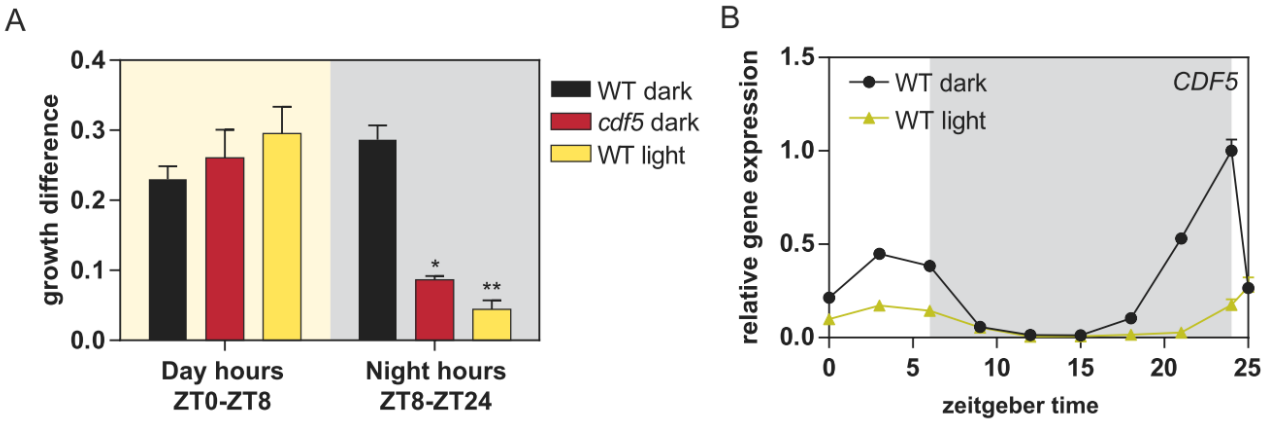


Figure 61. CDF5 induces growth during the night period of short days. (A) Quantification of hypocotyl elongation during the third day of growth of WT (WT dark) and *cdf5* (*cdf5* dark) mutant seedlings grown in SD, or WT seedlings grown for 2 days in SD and then transferred to continuous light (WT light). Data are presented as the growth difference during the day hours (ZT8-ZT0) or the night hours (ZT24-ZT8). Data are the means ± SE of two biological replicates, with at least 40 seedlings each. Asterisks indicate statistically differences from WT by Student's *t* test. **(B)** Expression of *CDF5* analyzed by qRT-PCR. Samples were taken during the third day under SD conditions (WT dark) or free running conditions (WT light) at the specified time points. Expression levels relative to WT at 24 h in SD are shown. Data are the means ± SE of technical triplicates.

4.4 CDF5 expression is directly regulated by PIFs.

Because diurnal and photoperiodic regulation of hypocotyl length is a PIF-dependent mechanism (Nozue *et al.*, 2007; Niwa *et al.*, 2009; Nusinow *et al.*, 2011; Soy *et al.*, 2012; Soy *et al.*, 2014), and *CDF5* is a PIF-induced gene in the dark (Figure 56), we checked whether the induction of *CDF5* at the end of the night in SDs is controlled by PIFs. First, we analyzed the expression of *CDF5* in *pifq* seedlings during the third day of growth in short days. The time course

experiment showed that *pifq* mutants did not induce *CDF5* expression at the end of the night in SDs to the same extent as WT seedlings do, and showed an expression pattern similar to seedlings grown in free running (Figure 62A). These data indicate that the peak of expression of *CDF5* in WT seedlings at the end of the night is dependent on the dark-induced accumulation of the PIF-proteins.

Next, in order to investigate the contribution of each PIF to this regulation, we analyzed *CDF5* expression in different *pif* mutant combinations at zeitgeber time 24 (ZT24) in SDs or in free running conditions. Because *pif3* had the strongest misregulation of *CDF5* among the *pif* mutants checked (Figure 62B), we concluded that PIF3 is the PIF with the most prominent role in regulating *CDF5* expression, although PIF4 and PIF5 had a significant contribution (Figure 62B). Moreover, available Chip-Seq data for PIF1, PIF3, PIF4 and PIF5 (Pfeiffer *et al.*, 2014) showed that the four PIFs are able to bind to the same promoter region of *CDF5*, in which we found multiple G-BOX and PBE binding motifs (Figure 62C), suggesting that PIFs directly and collectively induce the expression of *CDF5* at the end of the night.

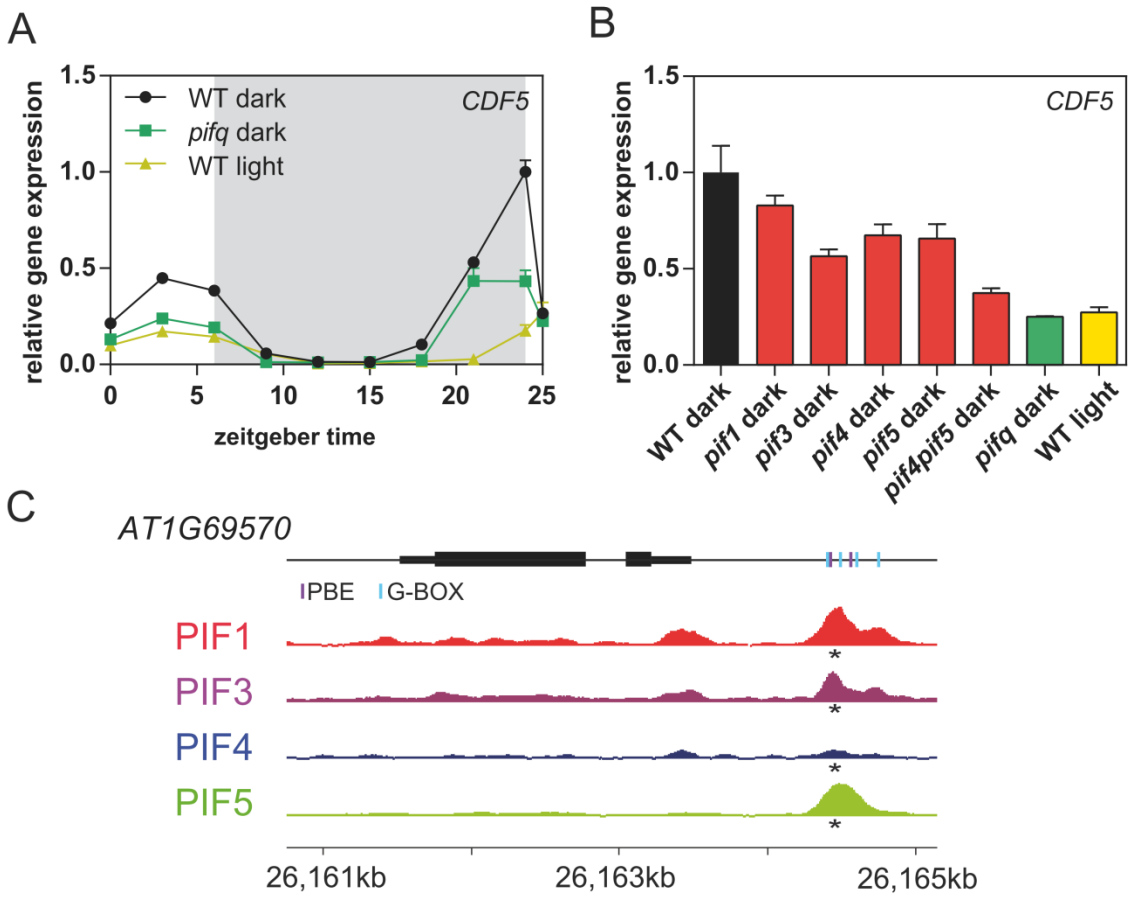


Figure 62. *CDF5* is induced at the end of the night in a PIF-dependent manner. Expression of *CDF5* analyzed by qRT-PCR. **(A)** Samples were taken during the third day in WT and *pifq* seedlings grown under SD conditions (WT and *pifq* dark), or in free running conditions (WT light) at the specified time points. Expression levels relative to WT at 24 h

in SD are shown. Data are the means ± SE of technical triplicates. **(B)** Samples were taken at ZT24 of the third day of growth under short days in different *pif* mutant combinations and WT seedlings (WT dark and *pif* mutants), or in WT seedlings grown in free running conditions during the third day (WT light). Expression levels are relative to WT dark. Data are the means ± SE of biological duplicates. **(C)** Visualization of ChIP-seq data in the genomic region encompassing the *CDF5* locus. Identified significant binding sites are indicated by an asterisk below the pile-up ChIP-seq tracks, and extends to 200bp around the centered peak summit defined as the binding-peak maximum. G- and PBE-box motifs in the promoter are indicated. Data obtained from (Pfeiffer *et al.*, 2014).

Together, the data suggest that PIFs directly induce the expression of *CDF5* at the end of the night to promote hypocotyl growth. To investigate the genetic interaction between CDF5 and PIFs, we generated a quintuple mutant (*pifqcdf5*) and analyzed the photomorphogenic phenotypes after 3 days under short day conditions. The result showed that *pifqcdf5* quintuple mutants have a similar phenotype compared to *pifq* mutants, and thus do not show an additive phenotype (Figure 63), suggesting that they probably act through the same molecular pathway(s).

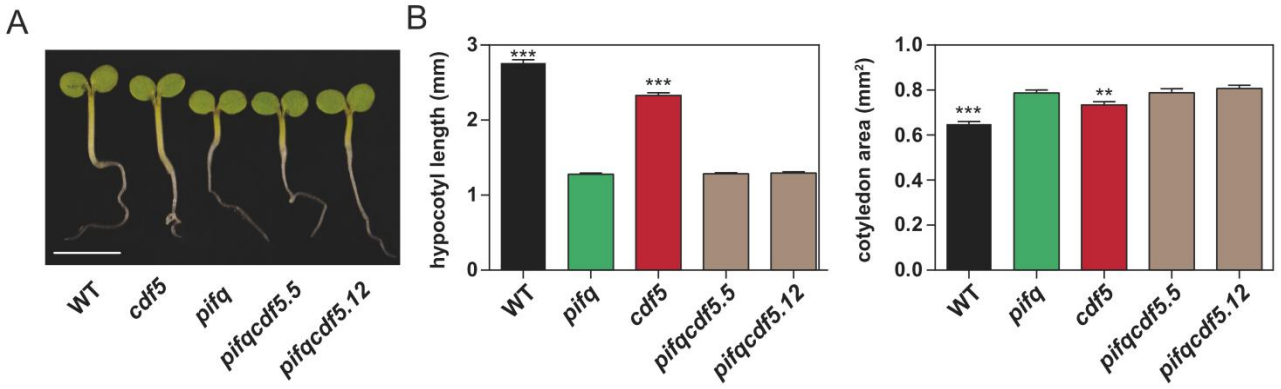


Figure 63. *cdf5* mutation in *pifq* background do not have and additive effect (A) Visual phenotype of WT, *cdf5*, *pifq* and two *pifqcdf5* mutants grown for three days in short days. Bar=2,5mm. **(B)** Quantification of hypocotyl length (left) and cotyledon area (right) of seedlings grown as in (A). Data are means ± SE of at least 40 seedlings and asterisks indicate statistically significant differences from *pifq* by Student's *t* test.

Importantly, *CDF5* overexpression in the *pifq* mutant background (*pifqCDF5OX* lines) under diurnal conditions caused a mild but significant suppression of the *pifq* photomorphogenic phenotype, with longer hypocotyls and reduced cotyledon area (Figure 64A). This result suggests that CDF5 acts downstream of PIF proteins to repress photomorphogenesis in short days. By contrast, no effect was observed in long days comparing the overexpressing *pifqCDF5OX* lines with *pifq* mutants (Figure 64A, 64B), similarly to the result obtained for *cdf5*CDF5OX seedlings grown in long days (Figure 60). Intriguingly, the effect of *CDF5* overexpression in repressing photomorphogenesis in SDs was less evident in the *pifq* background (Figure 64A) than in the *cdf5*

(Figure 60). Because these lines were generated independently, we need to test if these differences are due to different levels of CDF5 protein in each line, or alternatively because CDF5 needs the presence of PIFs to induce growth.

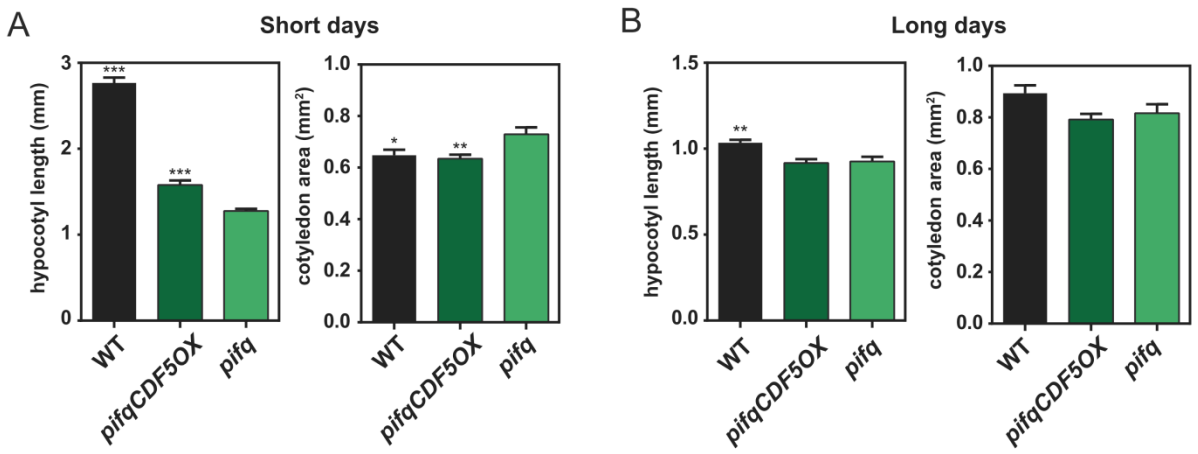


Figure 64. *pifqCDF5OX* lines partially suppresses the *pifq* phenotype in short days Quantification of hypocotyl length (left) and cotyledon area (right) of seedlings grown for three days in short days **(A)** or in long days **(B)**. Data are means ± SE of at least 20 seedlings and asterisks indicate statistically significant differences from *pifq* by Student's *t* test.

4.5 PRRs repress growth through the transcriptional repression of CDF5

PSEUDO-RESPONSE REGULATOR 5 (PRR5), PRR7 and PRR9 are transcriptional repressors in Arabidopsis circadian clock involved in the seasonal and diurnal regulation of growth, and they function to repress growth during the early night of short days (Nakamichi *et al.*, 2009; Niwa *et al.*, 2009; Farré and Liu, 2013). Accordingly, many phenotypic studies have revealed that *prr5, 7* and *9* mutants are longer than WT in short days (Nakamichi *et al.*, 2005; Nomoto *et al.*, 2012). In our SD conditions, we observed that *prr7* and *prr5* mutants are longer than *prr9*, indicating that among these PRRs, PRR7 and to less extent PRR5, are the most important regulators of growth (Figure 65A). Additionally, since ChIP-qPCR done with PRR5, PRR7 and PRR9 showed that they are able to bind directly to the *CDF5* promoter (Nakamichi et al., 2012), we hypothesized that the observed transcriptional repression of *CDF5* during early night was PRR dependent (Figure 61), providing a mechanism by which PRRs regulate rhythmic growth. Strikingly, we observed a strong upregulation of *CDF5* in *prr5prr7* double mutants at dusk (Figure 65B), which suggests that PRR5 and PRR7 repress growth during early night through the transcriptional repression of *CDF5*. To test this genetically, we generated *prr5cdf5* and *prr7cdf5* double mutants and we quantified the hypocotyl length of seedlings grown for four days in short days. Importantly, we observed that

prr5cdf5 and *prr7cdf5* double mutants were shorter than *prr7* and *prr5* single mutants (Figure 65C), providing a strong support to our hypothesis that transcriptional repression of *CDF5* by PRR5 and PRR7 is an important mechanism to regulate rhythmic hypocotyl growth in SDs. Overall, our results suggest that the precise transcriptional regulation of *CDF5* performed sequentially by the circadian clock at dusk and by light signaling at dawn, links both pathways to precisely regulate timing of growth under diurnal conditions.

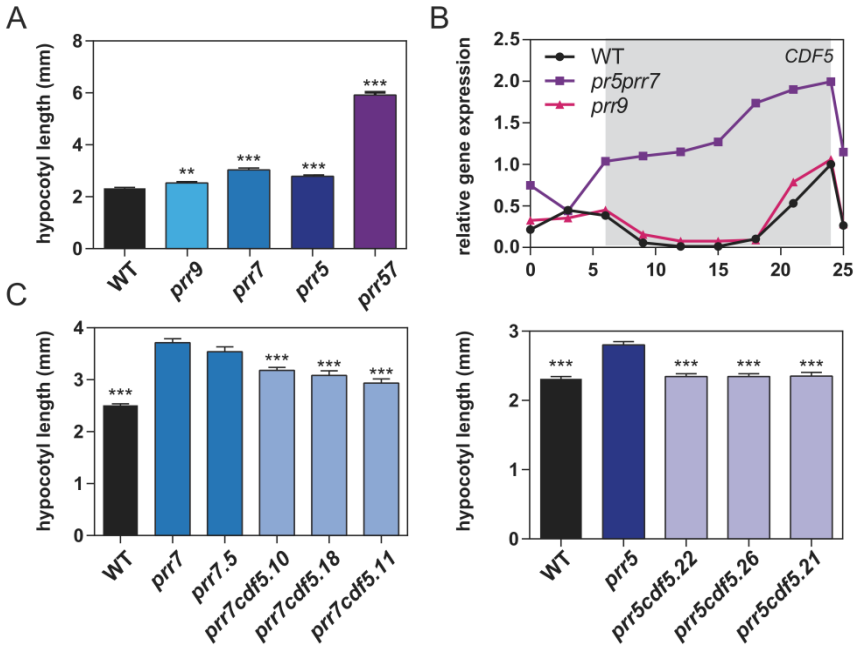


Figure 65. PRR5 and PRR7 repress growth through the transcriptional regulation of *CDF5*. (A) Quantification of hypocotyl length of *prr* single and double mutants grown for three days in short days. Data are means ± SE of at least 20 seedlings and asterisks indicate statistically differences from WT by Student's *t* test. **(B)** Expression of *CDF5* analyzed by qRT-PCR. Samples were taken during the third day in SD conditions in WT, *prr5prr7* and *prr9* mutants at the specified time points. Expression levels relative to WT at 24 h are shown. Data are the means ± SE of technical triplicates. **(C)** Quantification of hypocotyl length of *prr7, prr7cdf5* mutants (left) and *prr5, prr5cdf5* (right) grown for three days in short days. Data are means ± SE of at least 25 seedlings and asterisks indicate statistically differences from *prr* single mutants by Student's *t* test.

4.6 CDF5 regulation of cell expansion.

Diurnal growth depends on the capacity of hypocotyl cells to expand longitudinally (Farré, 2012), a process strongly regulated by plant hormones; consequently, the generation and action of these hormones vary over the course of the day (Michael *et al.*, 2008). Interestingly, many studies have demonstrated the interplay between auxin (IAA), PIF transcription factors and circadian clock in the control of hypocotyl growth (Nomoto *et al.*, 2012; Oh *et al.*, 2014). Under diurnal conditions,

PIFs regulate the expression of auxin genes modulating auxin-related pathways (Nozue *et al.*, 2011). In addition, both transcriptional and growth responses to exogenous auxin are gated by the clock, as seedling elongation is only enhanced when auxin is applied during the subjective night (Covington and Harmer, 2007). Therefore, we tested whether CDF5 is necessary for auxin-medaited induction of hypocotyl growth. We have performed preliminary experiments to test this hypothesis. Firstly, we have checked whether *CDF5* expression is regulated by auxin. Interestingly, using publicly available data we observed that *CDF5* mRNA levels are higher when auxins are applied (Nemhauser *et al.*, 2004; Oh *et al.*, 2014) (Figure 66A). To confirm auxin regulation, we need to perform this analysis in our short day conditions, and it would be very interesting to analyze the expression pattern of *CDF5* using mutants deficient in auxin synthesis, perception, or response. Next, experiments using seedlings grown for 11 days under short day conditions in plates containing 5µM of auxins compared to control plates, showed that *cdf5* mutants were not able to elongate in response to auxin (Figure 66B), suggesting that CDF5 may be necessary for auxin-mediated growth.

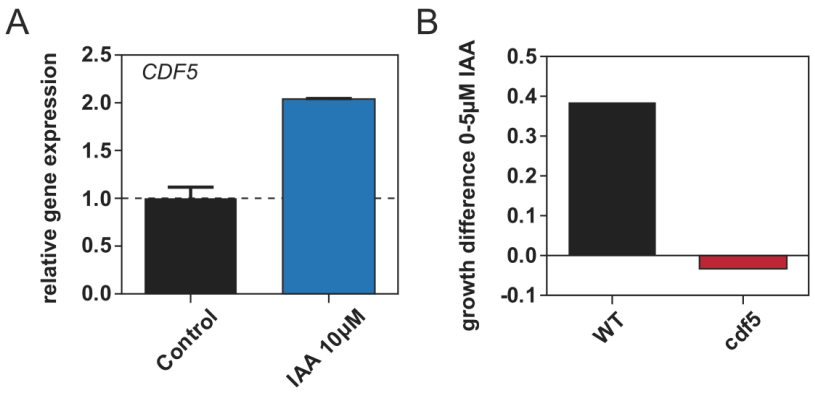
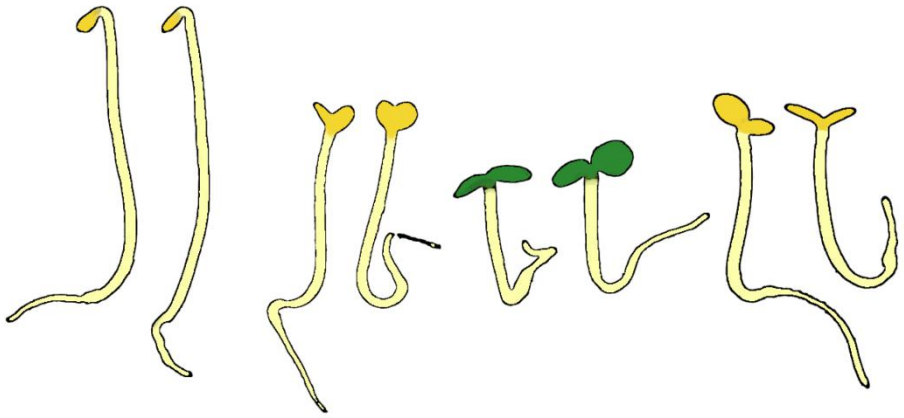


Figure 66. CDF5 is regulated by auxin levels to properly induce growth. (A) Expression of *CDF5* in five-day old light-grown Arabidopsis seedlings immersed 10 µM indole-3-acetic acid (IAA) or water (control). Expression levels relative to control are shown. Data is obtained from (Nemhauser *et al.*, 2004) **(B)** Quantification of hypocotyl growth in WT and *cdf5* 11 day-old SD-grown seedlings in the presence of 5µM auxin relative to the control plate. Data are means ± SE of at least 35 seedlings.

Interestingly, these preliminary results suggest that CDF5 might be necessary for the proper action of auxin in the regulation of cell expansion under short-day conditions. Extensive work must be done to validate this possibility.

DISCUSSION



1. A suppressor screen identifies new potential regulators of PIF-mediated seedling deetiolation.

At the beginning of my PhD project, available transcriptomic data of dark-grown *pifq* mutants lacking the PIF-quartet members PIF1, 3, 4 and 5 (PIFq), showed that these PIFs regulate the expression of ~10% of all genes in the dark. Moreover, the transcriptome of *pifq* dark-grown seedlings strongly resembles that of WT seedlings grown in the light, in agreement with the photomorphogenic phenotype of *pifq* seedlings (Leivar *et al.*, 2009). These and other published studies indicated that PIFs act mainly as transcriptional activators (Huq *et al.*, 2004; Al-Sady *et al.*, 2008; de Lucas *et al.*, 2008; Hornitschek *et al.*, 2009; Leivar *et al.*, 2009; Leivar and Quail, 2011). Given these results, we initially hypothesized that some of the downregulated genes in *pifq* (PIF-induced genes) could be responsible of the constitutive photomorphogenic phenotype of *pifq*. Therefore, we designed a gain-of-function genetic screen to identify these PIF-induced genes that repress seedling deetiolation in the dark. For this purpose, we mutagenized *pifq* mutant seedlings with the full-length cDNA overexpression (FOX) library (Figure 11) to identify suppressors of the constitutive photomorphogenic phenotype of *pifq* in the dark that restore the expression and function of these individual *pifq*-downregulated genes. Moreover, we reasoned that by using this system we could also identify skotomorphogenic inductors that belong to a PIF-independent pathway. An additional advantage of this gain-of-function approach is that it allows the identification of potential regulators that belong to gene families with redundant functions, a problem that is known to limit the success of loss-of-function screens.

Although our initial goal was to screen 20,000 *pifq* transformants, to cover twice the FOX library (Ichikawa *et al.*, 2006), we ended up screening only about 13,260 *pifq* transformants, covering the library about 1.3 times. The number of clones screened was enough to identify a large number of interesting potential suppressors, but because we did not obtain repeated clones, the screening was probably not saturated. As a result of the suppressor screen, we initially identified 28 validated regulator mutants that altered the hypocotyl and/or cotyledon phenotype of *pifq* (Table 1). Twenty of them corresponded to mutants that suppressed the *pifq* photomorphogenic phenotype (*sops*), whereas 8 corresponded to mutants showing an enhanced *pifq* photomorphogenic phenotype (*enops*). Although the identification of *enops* was not considered in our initial screen design, we selected them because they might also be important regulators of seedling deetiolation. Intriguingly, we only found mutants with an altered hypocotyl and/or cotyledon phenotype among the validated

suppressors, indicating organ-specific branching of the signaling pathway that implements photomorphogenesis. This view agrees with previous work from our laboratory showing that, rather than a pleiotropic effect, mutants of genes that regulate seedling deetiolation downstream of PIFs affect organ-specific branches of photomorphogenesis like hypocotyl, hook or cotyledons (Sentandreu *et al.*, 2011).

Interestingly, of the 28 validated mutants, we identified the single gene causing the suppressor/enhancer of *pifq* phenotype for 23 of them, whereas SOP 47 showed two cDNAs inserted (Table 1). These proportions were similar to those described for the FOX Agrobacterium library (Ichikawa *et al.*, 2006). Importantly, with the exception of *ELIP2* (Harari-Steinberg *et al.*, 2001), this genetic screen has allowed the identification of genes that have not been previously related to photomorphogenesis or PIF-regulated networks. Remarkably, their functional categorization shows that validated regulators are not restricted to a unique cell localization or biological process, consistent with the view that light signaling is a complex pleiotropic pathway (Figure 17). For example, the identified regulators include transcription factors that regulate nuclear gene expression (SOP 90), proteins localized to the endoplasmic reticulum involved in vesicle transport to the vacuole (SOP 46), or proteins that regulate the plastid gene expression (SOP 74). Moreover, some of them are proteins with unknown function, making them exciting candidates for future studies.

In addition, the functional categorization of the validated regulators shows an enrichment of genes encoding for chloroplast proteins. This intriguing result suggests that the chloroplasts play a role in regulating morphological features of seedling deetiolation through retrograde signaling, a possibility that we explored and confirmed in chapter 2. Consistent with the proposed model that chloroplast dysfunctions repress seedling deetiolation through retrograde signals (see below), we hypothesize that overexpression of SOPs encoding for chloroplast proteins are somehow producing defects in chloroplast biogenesis or function of dark-grown *pifq* mutant seedlings, which activates retrograde signals that revert the *pifq* constitutive photomorphogenic phenotype. We speculate that overexpression of these SOP genes generates an imbalance in the stoichiometry of proteins located in the chloroplast, which has been suggested to activate retrograde signals by a mechanism known as “control by epistasy of synthesis” (Wostrikoff *et al.*, 2004). Quantification of the expression of retrograde signaling gene markers in these *sop* mutant lines grown in the dark is an interesting experiment to start exploring this possibility.

Importantly, analysis of the expression pattern by available microarray data shows that all the identified PIF-regulated suppressor SOP genes are upregulated in the *pifq* (PIF repressed-genes), and in WT seedlings in response to light (Table 2). This result was somewhat surprising because, according to our screening design (Figure 11), we predicted to identify mainly PIF-induced genes repressed by light as skotomorphogenic inductors that, when overexpressed, suppress the *pifq* constitutive photomorphogenic phenotype. In addition, all the PIF-regulated enhancer *ENOP* genes are downregulated in *pifq* (PIF-induced genes), providing further complexity to the regulatory patterns identified. These apparent and unexpected contradictory patterns suggest a complex regulatory circuit acting downstream of the PIFs, where the balance between PIF-regulated positive a negative signals determine the photomorphogenic outcome (Sentandreu *et al.*, 2011). To better understand the role of each one of the identified *SOP* and *ENOP* genes in the light signaling pathway regulating seedling deetiolation, further characterization is needed. Moreover, in order to unambiguously confirm their role as regulators of photomorphogenic development, T2 seedlings must be first grown to obtain independent homozygous lines for each regulator in the T3, and then perform studies comparing these mutant lines with WT siblings to validate their phenotypes. In addition, expression of the inserted cDNA must be checked, to validate that they are being overexpressed in these lines. We followed this experimental design to study the role of *SOP 90*, a gene codifying the cycling DOF factor CDF2, in regulating photomorphogenesis. The results are shown in chapter 4.

2. Chloroplast status regulates photomorphogenic development.

Some publications in recent years have indicated that retrograde signals emitted by dysfunctional chloroplasts repress some aspects of photomorphogenesis such as pigment accumulation or chloroplast biogenesis (Larkin, 2014). Interestingly, effects in cotyledon aperture and expansion were suggested (Ruckle and Larkin, 2009), but further confirmation, as well as insights into the associated gene regulatory networks, are still missing. Remarkably, our work demonstrates that dysfunctions in chloroplast development suppress the morphological adaptations that occur after light exposure. Thus, successful chloroplast biogenesis can be considered not only as a consequence of the transcriptional remodeling that occurs in irradiated seedlings, produced in part by the proteomic degradation of PIFs, but also as a required process for photomorphogenesis, which represents a novel concept.

Curiously, although some work has identified that retrograde signals (RS) interact with light signaling networks (Ruckle *et al.*, 2012), this interaction has been focused mainly in the regulation of Photosynthetic Associated Nuclear Genes (PhANGs). Then, no clear connection has been made between developmental genes and their common regulation by retrograde and light signaling pathways. Strikingly, our work shows that the photomorphogenic repression produced when chloroplasts are damaged occurs through the same transcriptomic program as PIF regulation. Thus, retrograde signals regulate nuclear gene expression that promotes seedling deetiolation.

In addition, our work provides new tools to study the RS pathway because until now, mainly molecular phenotypes have been used. Moreover, our analysis combining phenotypic and gene expression studies allowed us to define among all the PIF-regulated genes (approximately 2.500 genes), a subset of 343 genes (PIF-RS genes) whose gene expression correlates with photomorphogenic phenotype in all tested conditions (Figures 27, 28, 31). Therefore, this association suggests that this subset could be responsible for the morphological changes that occur in response to light and retrograde signals. This study identifies a subset of genes that are seedling deetiolation effectors downstream of the PIF transcription factors, which advances our understanding of the transcriptomic networks that regulate seedling deetiolation.

2.1 RS and phy/PIF signalling pathways converge in the regulation of GLK1.

Interestingly, PIF-RS gene subset is enriched in GLK1-upregulated genes, which allow us to identify the GLK1 transcription factor as a key repressor of photomorphogenesis in the dark and in response to RS. Interestingly, GLK1 was reported as a transcription factor that induces chloroplast development through the transcriptional regulation of PhANGs (Waters *et al.*, 2009). In addition, some studies have shown that *GLK1* is downregulated by retrograde signals, which produces the transcriptional repression of PhANGs (Kakizaki *et al.*, 2009; Waters *et al.*, 2009). Interestingly, our work describes GLK1 as an important regulator of photomorphogenesis, and also provides a comprehensive picture of GLK1 function downstream of light and RS signals. We demonstrated that *GLK1* expression is strongly repressed in the dark, and this occurs through the direct regulation by PIF proteins (Figure 33B). At the same time, GLK1 plays a major role in retrograde signaling when chloroplasts are dysfunctional (Figure 37). *GLK1* is downregulated through an ABI4-independent, GUN1-mediated pathway (Figure 34), thereby suppressing photomorphogenic development. Importantly, the transcriptional network acting downstream of GLK1 to induce seedling morphogenesis after light exposure remains unknown (Figure 67).

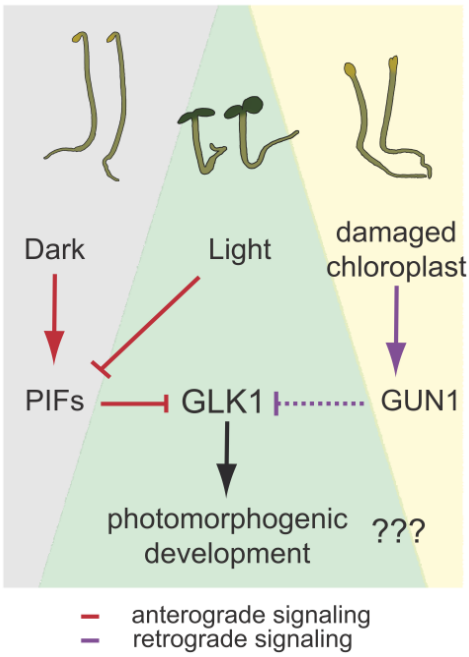


Figure 67. Proposed model depicting the antagonistic action on *GLK1* expression of light-regulated signaling mediated by the PIFs and GUN1-mediated RS from the chloroplast to regulate photomorphogenesis.

Furthermore, by comparing the PIF-RS and GLK1 regulated genes, we observed that only a subset of PIF-RS genes are defined as GLK1 upregulated genes (Figure 34B) (Waters *et al.*, 2009). We hypothesize that this incomplete overlap may be due to either or all of these reasons i) both experiments are done in different experimental conditions. ii) the *GLK1* microarray experiment, which was done with the glucocorticoid-inducible system that drives *GLK1* expression following treatment with dexamethasone (DEX), only identified a subset of all the GLK1-regulated genes since GLK1 induced levels are lower than endogenous levels; and/or iii) GLK1 is not the unique transcription factor repressing expression of light-induced PIF-repressed genes (Table 3). The incomplete suppression of photomorphogenesis in *glk1* light grown seedlings and in *GLK1OX* lincomycin treated seedlings; agree with the last option, supporting the idea that other transcription factors may be regulating photomorphogenesis downstream of light and retrograde signaling pathways.

Another interesting question that should be elucidated by future work is the molecular mechanism by which GUN1 downregulates *GLK1* in response to lincomycin. GUN1 is a Pentatricopeptide Repeat Protein (PPR) (Lurin *et al.*, 2004), and PRR proteins have been proposed to function in processing, editing, stability, and translation of RNA molecules, suggesting that the *GLK1* downregulation could imply regulation of RNA levels by direct interaction. In addition, GUN1 proteins are able to bind DNA (Koussevitzky *et al.*, 2007), which suggests that GUN1 can repress *GLK1* expression by direct binding to cis regulatory sequences in its promoter. Moreover, we cannot discard the possibility that GUN1 negatively regulates transcriptional activators of *GLK1* that have not yet been described.

2.2 Physiological implications of the molecular convergence of both signaling pathways.

Strikingly, regulation of *GLK1* by RS and PIFs reveals a novel regulatory node of physiological importance that allows seedlings to adjust nuclear gene expression to changes in light intensity during early deetiolation, when they may be especially sensitive to fluctuating light environments. It has been shown that plants have evolved a number of acclimation responses that effectively adapt the light-harvesting capacity of the photosynthetic apparatus to changes in illumination intensity, in order to prevent photodamage and oxidative stress (Pfannschmidt and Yang, 2012; Gordon *et al.*, 2013). Until now, the mechanisms described involved readjusting the expression of either nuclear photosynthesis genes to reconfigure the photosynthetic apparatus

(Pfannschmidt *et al.*, 2003), or of metabolic genes to adapt metabolic pathways (Bräutigam *et al.*, 2009). Here, we describe a new mechanism that allows plants acclimate to excess of light: when RS is activated in response to high light, seedlings adapt nuclear gene expression to delay deetiolation by reducing cotyledon aperture and hook opening to reduce the area exposed to light (Figure 45, 46).

We propose that the convergence of both stimuli -- high light (through retrograde signals) and darkness (by PIF transcriptional regulation) -- regulating the same transcriptional cascade is a useful mechanism because in the light, the phy/PIF system is not sensitive to differences between 'normal' light levels and potentially damaging high light. Therefore, chloroplasts become the light sensors in under high light conditions and signal to the nucleus through the same regulatory pathways than PIF/phy system to achieve similar phenotypic responses. This suggests an evolutionary scenario in which either RS or the PIF/phy system recruited the molecular pathway already in use by the other system. However, whether this molecular cascade was first used by RS or the PIF/phy system is still unknown.

Remarkably, in adult tissues retrograde signals induce senescence (Pogson *et al.*, 2008). Given that PIFs and GLKs have been recently related to the anterograde regulation of senescence (Song *et al.*, 2014), we propose that our convergence model may also be active during senescence.

3. BBX16 implements the morphological aspects of seedling photomorphogenesis downstream of GLKs.

Given that GLKs only have been described before as important regulators of chloroplast development as transcriptional activators of the expression of *PhANG* genes,. their role regulating morphological features of morphological photorphogenesis is completely unknown. Hence, the transcriptional cascade that implements cotyledon aperture and inhibits hypocotyl elongation remains unexplored. Because of the interesting enrichment of members of subclass III of BBX transcription factors among the GLK-regulated genes, we focused on their role in regulating photomorphogenesis downstream of the GLKs. Accordingly, some members of this family of transcription factors have been described either as positive and negative regulators of photomorphogenesis (Gangappa and Botto, 2014), and BBX16, one of the BBXs that is GLK upregulated (Waters *et al.*, 2009), is involved in phytochrome B-mediated shade avoidance responses, mainly regulating branching (Wang *et al.*, 2013; Zhang *et al.*, 2014).

3.1 BBX16 function regulating morphological photomorphogenesis

Interestingly, we found that *glk1*, *glk2* and *glk1glk2* mutants, which are hyposensitive to light, have less expression levels of the four members of class III *BBX* family (BBX14, BBX15, BBX16 and BBX17). This result suggested that GLKs induce photomorphogenic development through the transcriptional activation of these BBXs. Importantly, our experiments showed that among the members of class III, BBX16 is the BBX member with the strongest misregulation in *glk* mutants grown in the light (Figure 50), suggesting that BBX16 is the one that play the most prominent role in inducing photomorphogenesis downstream of the GLKs. Moreover, the stronger downregulation of *BBX16* observed in *glk2* mutant is in line with the stronger disruption of photomorphogenesis in *glk2* seedlings in the light compared to *glk1* seedlings. However, because of the mild phenotype of the *bbx16* mutant (Figure 51), and given that BBX14 and BBX15 are also strongly regulated by GLKs in the light, we reasoned that these other members of the class III may regulate seedling deetiolation redundantly with BBX16.

Importantly, we observed that GLK1 role in suppressing cotyledon aperture and in inducing hypocotyl elongation in response to retrograde signals is also dependent of the downregulation of *BBX16*. Interestingly, GLK2 does not regulate either the expression of *BBX16* in these conditions or

morphological photomorphogenesis. Therefore, *BBX16* transcriptional regulation is an interesting mechanism by which GLK1 specifically regulate morphological features of photomorphogenesis in response to retrograde signals. Moreover, transcriptional regulation of *BBX16* by both GLKs after light exposure agrees with the fact that under light conditions, GLK1 and GLK2 collectively regulate morphological photomorphogenesis. In the future, it will be interesting to determine the mechanism by which GLK2 regulate *BBX16* gene expression only in response to light stimulus and not to RS.

3.2 BBX16 function regulating chloroplast development

Importantly, only morphological photomorphogenesis is affected in *BBX16OX* seedlings grown with lincomycin, since the expression of *LHCB* genes is not misregulated in these seedlings (Figure 53, 54). This result suggests that BBX16 do not regulate chloroplast development in response to retrograde signaling. The specificity of BBX16 function, together with the fact that GLK2 does not regulate *BBX16* expression in these conditions, correlate with the role of GLK2 repressing chloroplast development in response to retrograde signals (Figure 41) (Waters *et al.*, 2009). Moreover, this result allows us to elucidate the function of GLK1 regulating photomorphogenesis (Figure 68), indicating that photomorphogenic signaling branches out downstream of GLK1. On the one hand, GLK1 directly regulates the expression of genes that forms the photosynthetic apparatus, inducing chloroplast functionality (Waters *et al.*, 2009). On the other hand, we have shown that GLK1 induces phenotypic changes to improve light perception: inhibition of hypocotyl elongation and cotyledon aperture, at least in part, through the transcriptional regulation of *BBX16*.

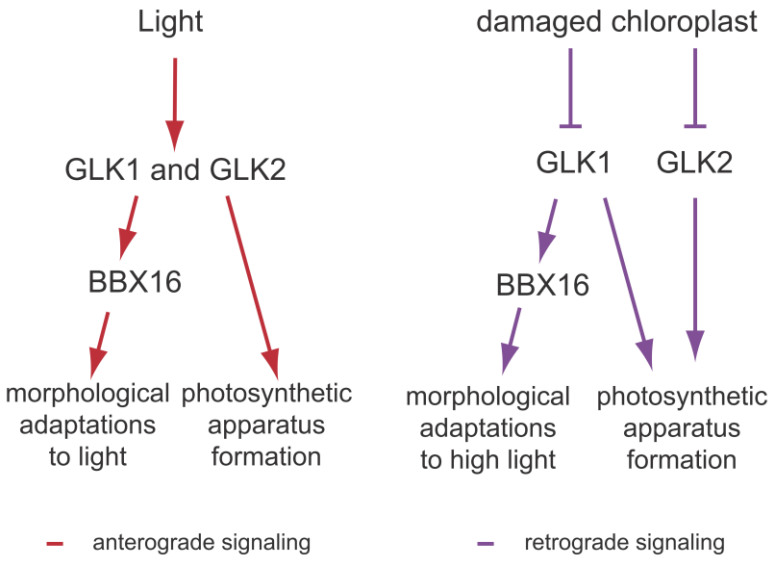


Figure 68. GLKs regulate different aspects of photomorphogenesis, in response to different stimulus. Proposed model depicting the role of GLKs regulating separately the formation of photosynthetic apparatus, by direct regulation of genes that codifies for components of photosynthetic apparatus, and inducing phenotypic adaptations to improve light perception through the transcriptional regulation of *BBX16*. GLK2 specifically regulate *BBX16* gene expression in response to light stimulus, in contrast, GLK1 also regulates *BBX16* in plates containing lincomycin.

Together, our work a) identifies the role of GLKs in the regulation of the overall photomorphogenic developmental program in response to RS (Chapter 2), b) describes the mechanism underlying how GLKs regulate photomorphogenesis downstream of PIFs and RS, and c) characterizes BBX16 as a downstream effector of GLK to specifically regulate morphological morphogenesis in response to retrograde signals (GLK1) and in response to light stimulus (GLK1 and GLK2).

4. CDF5 links clock and light signaling to regulate rhythmic growth.

SOP90, the suppressor mutant overexpressing *CDF2*, was one of the SOPs with strongest suppressor phenotype (Figure 55). In addition, comparison of transcriptomic data from *pifq* and WT seedlings showed that *CDF2*, as well as the two most related genes *CDF3* and *CDF5*, were PIF-regulated genes (Leivar *et al.*, 2009; Lorrain *et al.*, 2009; Nozue *et al.*, 2011). Moreover, *CDF2*, *CDF3*, and *CDF5* were described as PIF-bound genes in at least one ChIP-seq experiment performed with PIF proteins (Oh *et al.*, 2009; Hornitschek *et al.*, 2012; Oh *et al.*, 2012; Zhang *et al.*, 2013). Despite the fact that we started studying the role of CDFs because we found CDF2 in our suppressor screening, we decided to focus on CDF5 because the mutant *cdf5* was the one with the strongest disruption of photomorphogenic development in short days and under red light (Figure 57). In addition, we considered *CDF5*, a PIF-induced gene, the most interesting CDF because PIF's regulation of *CDF5* correlates well with its role repressing photomorphogenesis, and inducing growth at the end of the night in short days. In contrast, CDF2 and CDF3 are also repressors of photomorphogenesis, but are downregulated by PIFs (Figure 56). This pattern of regulation implies a more complex mechanism by which PIFs regulate photomorphogenic development through CDF2 and CDF3. One possibility is that they act during the first hours of light exposure to fine tune seedling deetiolation, avoiding over-responses to light (Sentandreu *et al.*, 2011). Further characterization is needed in order to validate this or other possibilities, and fully understand the role of CDF2 and CDF3 in the PIF transcriptional network.

4.1 CDF5 role in the photoperiodic regulation of growth

It is well determined that hypocotyl elongation in diurnal conditions is regulated in a seasonal manner, because it is a short-day-specific event regulated by the duration of the dark period of the day (Niwa *et al.*, 2009). In addition, growth is also regulated daily, being a time-of-day specific event that occurs at dawn in seedlings grown in short days (Nozue *et al.*, 2007). In recent years, much effort has been put to unravel the molecular mechanisms that regulate the photoperiodic plant growth. The results have established that both the circadian clock and the light signaling pathway regulate rhythmic growth in a coordinated manner (Dowson-Day and Millar, 1999; Farré, 2012). This diurnal and photoperiodic control of plant growth is best explained by the accumulation of PIF proteins at the end of the night specifically under short day conditions (Niwa *et al.*, 2009; Nomoto

et al., 2012; Nusinow *et al.*, 2012; Soy *et al.*, 2012; Yamashino *et al.*, 2012; Soy *et al.*, 2014). Our results demonstrate the importance of CDF5 accumulation to induce growth under these conditions, identifying a PIF-target that mediates PIF activity in implementing growth. Moreover, our data showing that both light and the circadian clock regulate *CDF5* accumulation represents a new molecular mechanism underlying rhythmic growth.

4.1.1 Daily regulation.

We have shown that *CDF5* is strongly regulated at the transcriptional level in short days, having a peak of expression at the end of the night, which contributes to hypocotyl elongation (Figure 61). Moreover, our results demonstrate that PRRs (circadian clock components) and PIFs contribute to implement this expression pattern (Figure 62, 65). PIFs collectively induce *CDF5* transcription at the end of the night; in addition, PRR5 and PRR7 repress the transcription of *CDF5* during early night. Remarkably, it is known that PIFs and PRR7 and PRR5 bind to the same region of the *CDF5* promoter, in which we found diverse G-BOX and PBE binding motifs (Nakamichi *et al.*, 2012; Pfeiffer *et al.*, 2014). This coincidence in the binding site of both groups of transcription factors suggest a mechanism that allows plants to ensure that *CDF5* is not expressed in the early night, when PIFs are only starting to accumulate and PRR7 and PRR5 are active. In contrast, PRR9 is active in the light hours of the day (Matsushika *et al.*, 2000) when PIF activity is minimum, and our results show that it is not involved in regulating *CDF5* expression. Because PRR9 also binds to the same binding region than PRR5 and PRR7, our data suggest an intrinsic difference among PRRs that provide specificity to their targets.

4.1.2 Seasonal regulation.

Moreover, although we have not checked CDF5 protein behavior in our seedlings in SD, we can speculate about the accumulation pattern based on previous results. It is well described that protein accumulation of the CDFs is regulated in a seasonal manner, because in the late evening of long days complexes formed by GIGANTEA and FKF1 induce their proteomic degradation (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Andres and Coupland, 2012). Accordingly, the phenotype of our *CDF5* overexpressing and deficient seedlings was much more evident in short days than in long days (Figure 60, 64). In addition, transcriptional levels of *CDF5* are also regulated in a seasonal manner through the gene expression regulation of their transcriptional inducers (PIFs), because light and circadian clock allow lower PIF accumulation in LD compared to SD (Niwa *et al.*, 2009; Nomoto *et al.*, 2012; Nusinow *et al.*, 2012; Soy *et al.*, 2012; Yamashino *et al.*, 2012; Soy

et al., 2014), which correlate with the expression levels of *CDF5* transcript (Figure 70) (Fornara *et al.*, 2009).

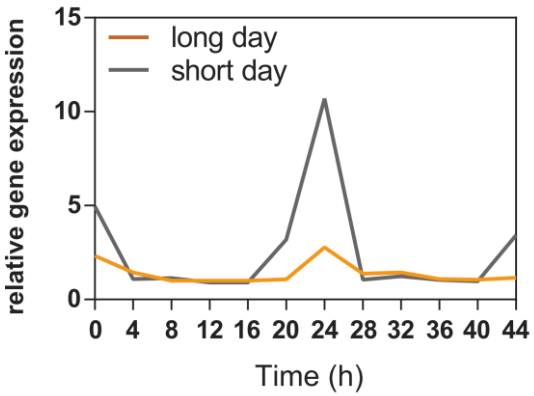


Figure 70. Short day and long day *CDF5* expression. The graph represents the expression values of *CDF5* in short day and long day conditions at the specified time points. Data obtained from diurnal (http://diurnal.mocklerlab.org/). Expression levels relative to long day at 12 h are shown.

4.1.3 Biological function of CDF5.

Our results indicate that, in short days, *CDF5* is directly regulated by PIFs at the end of the night to induce growth (Figure 61, 62). Interestingly, our data also suggest that auxin, a phytohormone that promotes growth, induces the expression of *CDF5* (Figure 66). Additionally, previous data have revealed that the auxin-response factor ARF6, and the brassinosteroid-signaling transcription factor BZR1, interact with each other and cooperatively regulate large numbers of common target genes to induce cell elongation and then induce growth. However, their DNA-binding activities are blocked by the gibberellin-inactivated repressor RGA (Oh *et al.*, 2014). Interestingly, among these commonly regulated genes we found *CDF5*, suggesting that their effect on the transcriptional regulation of *CDF5* may be relevant to implement cell expansion and, thus, hypocotyl elongation. Moreover, under diurnal conditions, growth is known to occur only at a specific time of the day due to the circadian modulation of hormone and light signal (a process known as "gating") (Covington and Harmer, 2007; Michael *et al.*, 2008; Niwa *et al.*, 2009; Arana *et al.*, 2011; Mizuno *et al.*, 2015). Given that our data indicate that PRR5 and PRR7, central components of the circadian clock, repress *CDF5* during the early night to restrict the period in which CDF5 is inducing growth, we reasoned that *CDF5* may be regulated by the circadian clock, as well as hormone and light stimuli to regulate the gating of hypocotyl growth response. Future work must be done to confirm this hypothesis.

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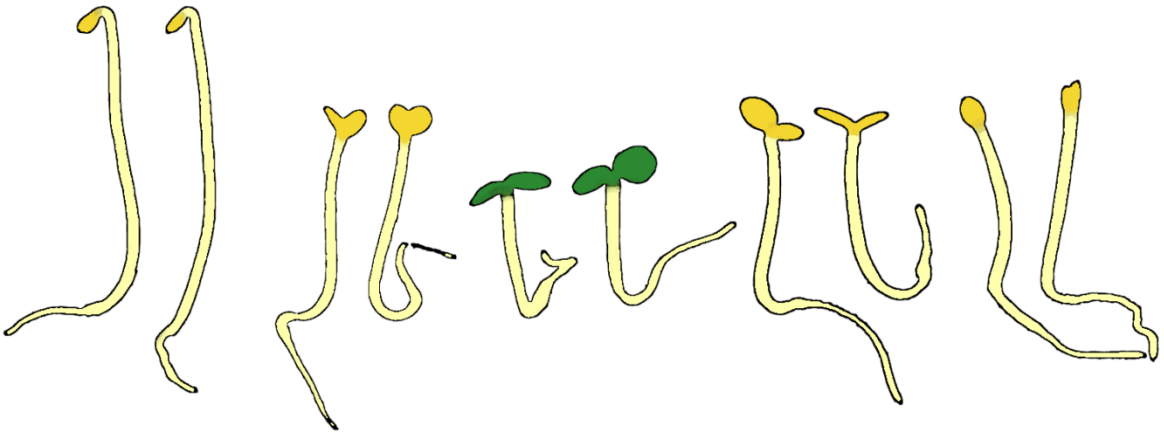
CONCLUSIONS



CONCLUSIONS

The conclusions of this thesis are:

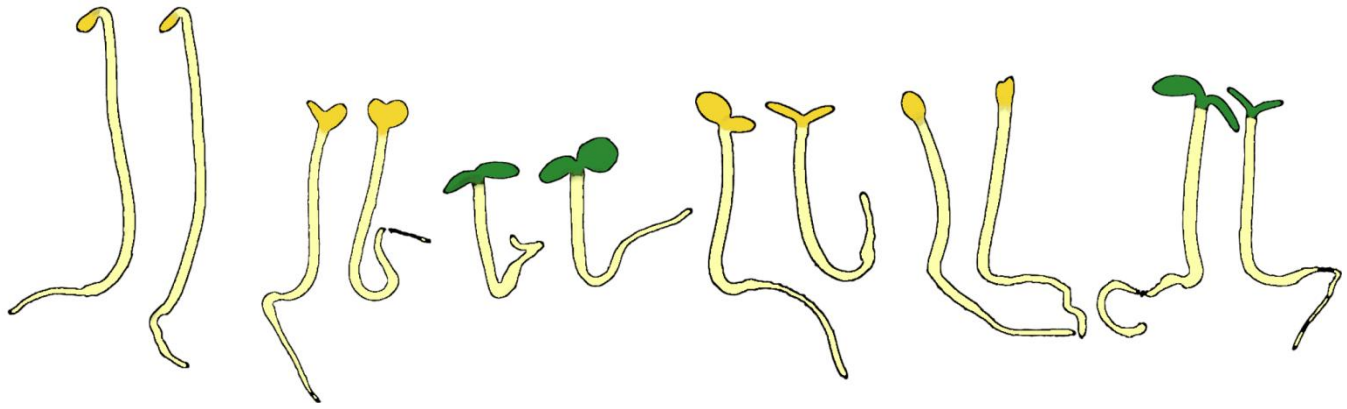
1. We have identified new regulators of photomorphogenesis acting downstream of the PIF transcription factors.
   - 1.1 Our genetic screen has allowed the identification of 23 genes that suppress or enhance the *pifq* constitutive photomorphogenic phenotype when overexpressed.
   - 1.2 Some of the effector genes identified in our genetic screen belong to the PIF transcriptional network, and include PIF-regulated genes in different experimental conditions tested, and/or previously described PIF-bound genes.
   - 1.3 Transcriptional regulation of *CDF5* by the circadian clock and the PIF transcription factors defines a novel mechanism of regulation of growth under diurnal conditions.
     - 1.3.1 CDF5 induces hypocotyl growth and inhibits cotyledon expansion in red light and under diurnal conditions.
     - 1.3.2 *CDF5* expression is directly induced by the PIFs at the end of the night, when it may facilitate the auxin action in regulating cell expansion[gm1] . During the early part of the night, *CDF5* is transcriptionally repressed by PRR5 and PRR7 to inhibit hypocotyl elongation.

2 Dysfunctions in chloroplast biogenesis repress photomorphogenesis in order to adjust seedling development to the photosynthetic capacity of the chloroplast.

   - 1.1 A disruption in chloroplast biogenesis suppresses PIF-mediated photomorphogenesis through retrograde signaling.
   - 1.2 GUN1-mediated retrograde signaling represses expression of light induced-PIF repressed genes.
   - 1.3 GLK1, a direct PIF-repressed target,induces photomorphogenesis and is a GUN1-repressed gene. Thus, GLK1 functions as a link between both the light/PIF and the retrograde signaling pathways.
   - 1.4 GLK1 induces the expression of *BBX16* in order to promote the morphological features of photomorphogenesis in the light and in response to retrograde signaling. In contrast, GLK2 is only able to regulate morphological photomorphogenesis in the light, also through the transcriptional regulation of *BBX16*.

1.5 *BBX16* is a transcription factor that induces photomorphogenesis.

Material and Methods



Materials and Methods

1. Plant material and growth conditions

Arabidopsis thaliana wild-type and mutant seeds used in this study were all in the Columbia genetic background. The mutant backgrounds, transgenic lines and high order mutants generated in this work are listed below in Table 4.

Table 4: Mutant and transgenic lines used in this PhD thesis. For each mutant or transgenic line used in this work, columns indicate the mutant name used in the PhD thesis, the AGI gene number (AGI), the mutant allele, and the code of the European Arabidopsis stock center (NASC code). The reference for the mutants and transgenic lines published elsewhere is indicated in the last column.

Mutant or trasgenic line	**AGI**	**Allele**	**NASC code**	**References**
pifq		*pif1-1 pif3-3 pif4-2 pif5-3*		(Leivar *et al.*, 2009)
glk1	AT2G20570	*Atglk1.1*	N9805	(Fitter *et al.*, 2002)
glk2	AT5G44190	*Atglk2.1*	N9806	(Fitter *et al.*, 2002)
glk1glk2		*Atglk1.1;glk2.1*	N9807	(Fitter *et al.*, 2002)
GLK1OX	AT2G20570	*35S::GLK1*	N9905	(Waters *et al.*, 2008)
GLK2OX	AT5G44190	*35S::GLK2*	N9906	(Waters *et al.*, 2008)
CGA1OX	AT5G56860	*35S::CGA1*		(Chiang *et al.*, 2012)
abi4	AT2G40220	*abi4t*	N580095	(Shu *et al.*, 2013)
gun1	AT2G31400		N866021	
bbx16	AT1G73870	*col7*		(Wang *et al.*, 2013)
BBX16OX 10	AT1G73870	*col7 OX 10*		(Wang *et al.*, 2013)
BBX16OX 11	AT1G73870	*col7 OX 11*		(Wang *et al.*, 2013)
cdf2	AT5G39660		N812792	
cdf3	AT3G47500		N862942	(Khanna *et al.*, 2006)
cdf5	AT1G69570	*cdf5-1*	N576153	(Fornara *et al.*, 2009)
prr5	AT5G24470	*prr5-1*	N506280	(Eriksson *et al.*, 2003)
prr7	AT5G02810	*prr7-3*	N530430	(Salomé and McClung, 2005)
prr9	AT2G46790	*prr9-1*	N657486	(Eriksson *et al.*, 2003)
prr5prr7		*prr5-1prr7-3*		
cdf2cdf3		generated by crossing *cdf2* and *cdf3* mutant backgrounds.		
cdf2cdf5		generated by crossing *cdf2* and *cdf5* mutant backgrounds.		
cdf3cdf5		generated by crossing *cdf3* and *cdf5* mutant backgrounds.		
cdf5pifq		generated by crossing *cdf5* and *pifq* mutant backgrounds.		
cdf5prr5		generated by crossing *cdf5* and *prr5* mutant backgrounds.		

cdf5prr7		generated by crossing *cdf5* and *prr7* mutant background.
cdf5CDF5OX		generated by transforming *cdf5* mutants with 35:CDF5:GFP construct.
pifqCDF5OX		generated by transforming *pifq* mutants with 35:CDF5:GFP construct.

Seeds were surface-sterilized in 20% bleach and 0.03% Triton X-100 for 10 min and plated on 0.5X Murashige and Skoog (MS) without sucrose, stratified at 4°C in the dark for 4 days, exposed to white light for 3 h to induce germination, and then placed to the specific light conditions indicated in each experiment. For experiments done under continuous light conditions, plates where usually placed under continuous white light (1 $\mu mol \cdot m^{-2} \cdot s^{-1}$), red light (1.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$), or darkness for three days unless otherwise indicated. In the case of the experiment shown in Figure 43, a white light intensity of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was used. High light experiments shown in chapter 2 were performed using a combination of red (60%) and blue (40%) light, and a fluence rate of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Light conditions) or 310 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (High light conditions). Fluence rates were measured according to (Martínez-García *et al.*, 2014). For experiments done in photoperiodic conditions, seedlings were grown in short day (8h white light ($84 \mu mol \cdot m^{-2} \cdot s^{-1}$) and 16h dark), long-day (16h white light ($84 \mu mol \cdot m^{-2} \cdot s^{-1}$) and 8h dark) or continuous light ((24h white light ($84 \mu mol \cdot m^{-2} \cdot s^{-1}$)) for the time indicated in each experiment. In the case of screening and validations of putative regulators of seedling deetiolation, plates were placed in the dark for 2days plus 17 hours after stratification. For drug treatments, media was supplemented with 0.5 mM lincomycin (Sigma L6004) (Sullivan and Gray, 1999b), 5µM IAA (Sigma-Aldrich) or $30 \mu g \cdot ml^{-1}$ of Hygromycin.

2. Phenotypic measurements and statistical analysis

Hypocotyl length, cotyledon area, cotyledon aperture and hook unfolding were measured as described (Sentandreu 2011), by using NIH Image software (Image J, National Institutes of Health). 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. Mean and standard error were calculated from at least 25 seedlings, and statistically analysis was performed by the Student's *t* test (homoscedastic, two-tailed distribution) in Excel (Microsoft). Statistically significant differences between lines were defined as those with a P value < 0.05. In addition, statistical analysis of the morphological data shown in Figures 25, 30, 37 and 41 was performed by one-way ANOVA, and the differences between means were evaluated using Tukey-b post-hoc multiple comparison test (IBM SPSS Statistics Software). Statistically significant

differences were defined as those with a P value < 0.05. Asterisks in the figures indicate significance level (P < 0.05 (*); P < 0.01 (**); P < 0.001 (***)).

3. Molecular cloning of FOX library cDNAs

Details on the materials and methods used for the genetic screen to search for suppressors of *pifq* mutants are provided in the results section. To confirm the presence and identity of the full length cDNA from FOX library responsible for the phenotype of the *sop* and *enop* mutants, we first obtained the genomic DNA from each mutant. We then amplified the FOX cDNA by using primers EMP 401 (5'-ATGAAAAAGCCTGAACTCACCG-3') and EMP 402 (5'-TCGAGAGCCTGCGCGACG-3') that annealed with 5' and 3' regions in the pBIG2113SF vector (Ichikawa *et al.*, 2006). PCR (Polymerase Chain Reaction) conditions were 94°C for 2 min, and 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 5 min. Once we obtained the PCR product, we purified it using the QIAquick Gel extraction kit (Qiagen), and cloned in a TA-TOPO vector (Invitrogen). The resulting construct was amplified and purified using the High Pure Plasmid Isolation Kit (Roche), and the cloned cDNA was then sequenced.

4. Molecular cloning and generation of transgenic lines overexpressing CDF5

Transgenic lines for overexpression of CDF5 were generated by *Agrobacterium*-mediated transformation of *cdf5* and *pifq* backgrounds. 35S::CDF5:GFP fusions were generated in pH7FGW2 destination vector by using the recombination Gateway Cloning system. First, *CDF5* cDNA was amplified by RT-PCR from from 3d-old dark-grown *pifq* seedlings using primers that recognize and contain the recombination sites that are needed for Gateway system: EMP 536 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTAAATCTAGAGAT-3') and EMP 537 (5'- GGGGACCAGTTTGTACAAGAAAGCTGGGTCTTGTTGCATGCTCTCCCTGAA -3'). The purified PCR product was placed into the Gateway donor vector pDONOR221 by the BP reaction, by mixing 100 fmol of PCR product, 150 ng of pDONOR221, and 1µl of the BP clonase enzyme diluted in 5µl of TE pH 8.0. CDF5 cloned in pDONOR221 was then isolated and sequenced. Recombination LR reaction was subsequently performed by mixing the CDF5-pDONOR construct (150ng/µl), the pH7FGW2 destination vector (300ng/µl), and 1µl of the LR clonase enzyme diluted in 5µl of TE buffer pH 8.0. All recombination reactions were incubated for 2 hours at 25ºC and treated for 10 minutes at 37ºC with Proteinase K (Invitrogen). CDF5-

pH7FGW2 construct was then isolated and sequenced, and it was introduced into Agrobacterim GV3101 strain by using a standard heat shock procedure. Agrobacterium strain carrying the plasmid was selected on plates with rifampicin and spectinomycin. Agrobacterium-mediated plant transformation was done as described (Clough, 2005).

5. Quantitative Reverse Transcriptase (qRT-PCR)

For qRT-PCR analysis, seedlings were grown in the dark or in white light for the indicated time. qRT-PCR was performed as described previously (Khanna *et al.*, 2007) with variations. Briefly, 10µg of total RNA extracted with the RNeasy Plant Mini Kit (Qiagen) was treated with DNase I(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 2µl of this mix was used for real-time PCR (Light Cycler 480; Roche) using SYBR Premix Ex Taq (Roche) and primers at a 300nM concentration. Gene expression was usually measured in three independent biological replicates, and at least three technical replicates were done for each of the biological replicates. *PP2A* (*AT1G13320*) was used for normalization as described (Shin *et al.*, 2007). Primers sequences used for qRT-PCR are described in Table 5.

Table 5: List of primers used for qRT-PCR. For each gene analyzed by qRT-PCR the pair of primer sequences is shown. Columns indicate the primer name, the sequence, and the gene amplified. For primers described elsewhere, the reference is indicated.

Primer	Sequence	Gene	Reference
EMP628	GCTACGAGATTTAGAGCACCG	*GLK1*	
EMP629	TTGACGGATGTAAGTCTACCG	*GLK1*	
EMP630	GGATATGGTATGGCATCATCG	*GLK2*	
EMP631	GCATCTATGCTCTCATTCGAG	*GLK2*	
EMP634	TGAGTATATTGACTGGCTGGG	*GUN1*	
EMP635	GCATTTTGACAGGTGGAATGG	*GUN1*	
PLR 84	CGTCCCCGGAAAGTGAGTT	*LHCB 1.4*	
PLR 85	TGCAACAAACCGGATACACAC	*LHCB 1.4*	
EMP945	GCTTTGTAAACTCGTGATTGTG	*LHCB 2.2*	(Woodson *et al.*, 2013)
EMP946	TGCCAAATTCACATCAAACG	*LHCB 2.2*	(Woodson *et al.*, 2013)
EMP957	TTTTTATCAATGGACCGCAAC	*BBX14*	
EMP958	GTGTTGCCAAATTACTGCTGC	*BBX14*	
EMP867	CCGTTGGTGTCTGAGCAGTTC	*BBX15*	
EMP868	TCAGCGTTGTCTTCCTCCTCC	*BBX15*	
EMP869	TTGGGATAATCACGGTTCGCC	*BBX16*	

EMP870	GTACCCACCCACAACATGAGG	*BBX16*	
EMP881	TGAGGGATTCACAGAGATGGG	*BBX17*	
EMP882	TCCAGGACATGCTTCAGCTC	*BBX17*	
EMP502	TTACCGTTTTACCCTCCACC	*CDF2*	
EMP503	GCATCCATGTGAAGCTGTTC	*CDF2*	
EMP517	TGAGAAGTGTTCCAATCGG	*CDF1*	
EMP518	TTCGGAGATAGTCACATGG	*CDF1*	
EMP521	TCGAAAAAGGACAACGAGAC	*CDF3*	(Fornara *et al.*, 2009)
EMP522	AGCAGAAAGAACAGGGGAGT	*CDF3*	(Fornara *et al.*, 2009)
EMP523	GGTATGCTTGGAGATGGAAA	*CDF4*	(Fornara *et al.*, 2009)
EMP524	CCCAATAGCAACCAAATTCA	*CDF4*	(Fornara *et al.*, 2009)
EMP525	TGGTCTCCGAAACTTCTCAC	*CDF5*	(Fornara *et al.*, 2009)
EMP526	GCAACTTCATCACACAATGG	*CDF5*	(Fornara *et al.*, 2009)

6. RNA-seq library construction and data processing

Extraction of total RNA from 3-d-old dark-grown seedlings and preparation of sequencing libraries were performed as described (Zhang *et al.*, 2013). Libraries from triplicate biological samples were assayed by 50-cycle single-end sequencing on an Illumina HiSeq platform. Reads were aligned to the TAIR10 representative transcriptome using Bowtie (Langmead *et al.*, 2009) with one mismatch allowed. Only reads mapping uniquely to the 3'-end 500-bp region of the coding strand were counted for gene expression. Differentially expressed genes were identified using the edgeR package (Robinson *et al.*, 2009) among those genes in which at least 2 of the 6 samples being compared had ≥ 5 reads per million. Statistically significant (SS) genes were defined as those that differ with a P value ≤ 0.05 (adjusted for false discovery rate, FDR) among samples, and SSTF (SS two fold) genes as those that differ by ≥2-fold with a P value ≤ 0.01 (adjusted for FDR).

7. Microarray data analysis

Gene expression data shown in Figures 18, 27, 28, 31, 35 and 39 were obtained from the published microarray experiments GSE5770 (Koussevitzky *et al.*, 2007) and GSE17159 (Leivar *et al.*, 2009). As a control, for each subset of genes analyzed, we compared the gene expression between the equivalent light-grown wild-type (WT Light) samples in the two independent microarray experiments. The absence of a statistically significant difference in gene expression between WT light samples allowed us to integrate the data from the two independent microarray experiments. Represented WT light values in Figures 27, 28, 35 and 39 are from (Koussevitzky *et al.*, 2007). Gene expression data shown in Figure 46B were obtained from GSE7743 (Kleine *et al.*, 2007), whereas data shown in Figure 66 were obtained from GSE863 (Nemhauser *et al.*, 2004).

Statistical analysis of the microarray expression data was performed by the Student's *t* test (homoscedastic, two-tailed distribution) in Excel (Microsoft). Statistically significant differences between lines were defined as those with a P value < 0.05. Asterisks in the figures indicate significance level ($P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***)).

8. Promoter analysis for DNA binding motifs

Analysis shown in Figure 32 for statistically overrepresented 6-mer motifs in the 500-bp genomic sequence upstream of the start codon of genes in gene-set PIF-RS was performed using the "Motif Analysis" tool available at The Arabidopsis Information Resource (http://Arabidopsis.org/tools/bulk/motiffinder/index.jsp). Hypergeometric test was performed using R.

9. Protein extraction and immunoblots

To perform the western blot shown in Figure 45, protein extracts were prepared from 2-day-old dark-grown WT and *gun1* seedlings transferred to white light for the time and light intensities indicated. Protein extracts were performed as described (Al-Sady *et al.*, 2006) in boiling extraction buffer (100 mM MOPS [pH 7.6], 5% SDS, 10% glycerol, 4 mM EDTA, 2 gl^{-1} aprotinin, 3 gl^{-1} leupeptin, 1 gl^{-1} pepstatin, and 2 mM PMSF). Total protein was quantified using a Protein DC kit (Bio-Rad), and β-mercaptoethanol was added just before loading. Aliquots from each sample containing equal amounts of total protein (150 μg) were subjected to 7.5 % SDS-PAGE gels. Proteins were then transferred to Immobilon-P membrane (Millipore), and immunodetection of endogenous PIF3 was performed as previously detailed (Soy *et al.*, 2012) using a rabbit anti-PIF3 polyclonal antibody (Al-Sady *et al.*, 2006). The membrane was stained with Coomassie blue as a loading control.

10. Transmission electron microscopy (TEM)

These experiments were performed at "Servei de Microscopia Electronica" of the Universitat de Barcelona in collaboration with Dr. Dolores Ludevid. Cotyledons from 3d-old dark-grown seedlings were fixed and processed as described in (Leivar *et al.*, 2009), except that a Leica EM PACT2-RTS high-pressure freezing machine (Leica Microsystems, Vienna, Austria) was used for the high pressure freezing (HPF) method. Ultrathin sections were visualized in a Jeol JEM1010

electron microscope (JEOL Ltd, Akishima, Tokyo, Japan). Images were recorded with a SIS Mega View III CCD camera.

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