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Authors	Martín, Guiomar;Veciana, Nil;Boix, Marc;Rovira, Arnau;Henriques, Rossana;Monte, Elena
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The photoperiodic response of hypocotyl elongation involves regulation of CDF1 and CDF5 activity

Guiomar Martín^{a,b,†}, Nil Veciana^{a,†}, Marc Boix^a, Arnau Rovira^a, Rossana Henriques^{a,c,d,*} and Elena Monte^{a,e,*}

^aCenter for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, Edifici CRAG, Bellaterra, 08193 Barcelona, Spain.

^bInstituto Gulbenkian de Ciência (IGC), 2780-156 Oeiras, Portugal

^cSchool of Biological, Earth and Environmental Sciences, University College Cork, Distillery Fields, North Mall. T23 TK30 Cork. Ireland.

^dEnvironmental Research Institute, University College Cork, Lee Road, T23 XE10 Cork, Ireland.

Consejo Superior de Investigaciones Científicas (CSIC), 08028 Barcelona, Spain.

[†]These authors equally contributed to this work

Correspondence

*Corresponding authors,

e-mails: elena.monte@cragenomics.es, rossana.henriques@ucc.ie

Hypocotyl elongation relies on directional cell expansion, a process under light and circadian clock control. Under short photoperiods (SD), hypocotyl elongation in *Arabidopsis thaliana* follows a rhythmic pattern, a process in which circadian morning-to-midnight waves of the transcriptional repressors PSEUDO-RESPONSE REGULATORS (PRRs) jointly gate PHYTOCHROME-INTERACTING FACTOR (PIF) activity to dawn. Previously, we described *CYCLING DOF FACTOR 5* (*CDF5*) as a target of this antagonistic PRR/PIF dynamic interplay. Under SD, PIFs induce *CDF5* accumulation specifically at dawn, when it promotes the expression of positive cell elongation regulators such as *YUCCA8* to induce growth. In contrast to SD, hypocotyl elongation under long days (LD) is largely reduced. Here, we examine whether *CDF5* is an actor in this photoperiod specific regulation. We report that transcription of *CDF5* is robustly induced in SD compared to LD, in accordance with PIFs accumulating to higher levels in SD, and in contrast to other members of the CDF family, whose expression is mainly clock regulated and have similar waveforms in SD and LD. Notably, when *CDF5* was constitutively expressed under LD, *CDF5* protein accumulated to levels comparable to SD but was inactive in promoting cell elongation. Similar results were observed for *CDF1*. Our findings indicate that both CDFs can promote cell elongation specifically in shorter photoperiods, however their activity in LD is inhibited at the post-translational level. These data not only expand our understanding of the biological role of CDF transcription factors, but also identify a previously unrecognized regulatory layer in the photoperiodic response of hypocotyl elongation.

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Abbreviations – SD, short day; LD, long day; PIF, phytochrome-interacting factor; PRR, pseudo-response regulator; CDF, cycling DOF factor; DOF, DNA binding with one finger; YUC8, YUCCA8; bHLH, basic helix-loop-helix; GI, gigantea; ELF3, early flowering 3; FKF1, flavin-binding, kelch repeat, F-box 1; FLORE, CDF5 long non-coding RNA; DAG1, DOF affecting germination 1.

Introduction

After the seed germinates, hypocotyl elongation occurs largely by cell expansion, and is highly sensitive to internal and environmental signals. The hypocotyl responds exquisitely to alterations in the quality, intensity, direction and duration of light, and has been a model organ for the elucidation of photosensory pathways and their integration with other cues such as the circadian clock, temperature, metabolic state or hormone homeostasis (Gray et al. 1998, Vandenbussche et al. 2005, Franklin et al. 2014, Gommers and Monte 2018, Simon et al. 2018).

In *Arabidopsis thaliana* seedlings grown under short photoperiods (SD), hypocotyl elongation is rhythmic and peaks at dawn coinciding with maximum accumulation and activity of the basic-helix-loop-helix (bHLH) transcription factors PHYTOCHROME-INTERACTING FACTORS (PIFs), which act in a partially redundant manner to promote growth (Nozue et al. 2007, Leivar and Monte 2014, Soy et al. 2014, Paik et al. 2017). Elongation rhythmicity under SD is the result of multi-level regulation of the timing of PIF protein accumulation and transcriptional activity. First, the clock gates *PIF4* and *PIF5* expression to the night period (Nozue et al. 2007, Nusinow et al. 2011). Second, the phytochrome photoreceptors impose oscillation of the PIF proteins, allowing accumulation during the night hours and targeting them for degradation in the light. PIF3, which is constitutively expressed across the 24 h, accumulates progressively during the night and is targeted for fast degradation by active phytochromes in the light, when growth is reduced (Nozue et al. 2007, Soy et al. 2012, Soy et al. 2014, Van Buskirk et al. 2014), a mechanism that is likely to also occur for PIF1 (Soy et al. 2014). Furthermore, PIF activity is gated by the direct interaction and antagonistic action of the core clock components and transcriptional repressors PSEUDO-RESPONSE REGULATORS (PRRs) (Soy et al. 2016, Martín et al. 2018), by GIGANTEA (GI) (Nohales et al. 2019), the evening complex (Nusinow et al. 2011) and EARLY FLOWERING 3 (ELF3) (Nieto et al. 2015), and regulated by brassinosteroid-dependent phosphorylation (Bernardo-García et al. 2014). Lastly, PIF directly induce expression of growth-promoting genes, including auxin signaling and other hormone-related genes (Michael et al. 2008, Nozue et al. 2011, Soy et al. 2016, Martín et al. 2018).

Hypocotyl elongation under diurnal conditions is regulated by photoperiod. Due to the clock and PIF interplay, there is a positive non-linear correlation of elongation with the length of the night (Niwa et al. 2009). Compared to SD, hypocotyls are distinctly shorter in long days (LD). Remarkably, and in contrast to SD, the growth peak in LD is shifted from dawn towards early morning (Nozue et al. 2007).

Recently, we have identified the DOF transcription factor *CDF5* (CYCLING DOF FACTOR 5) as a positive regulator of hypocotyl elongation under SD (Martín et al. 2018). *CDF5* promoter is a common direct target for antagonistic regulation by PRRs and PIFs. In the morning and through the middle of the night under SD, *CDF5* expression is maintained low by circadian morning-to-midnight repression by sequential waves of PRR accumulation PRR9, PRR7, PRR5, and TOC1, which are negative regulators of hypocotyl elongation. The PRRs inhibit PIF activity and gate PIF-promoted

CDF5 expression to pre-dawn hours, when PIF abundance peaks and coincides with low PRR presence. At dawn, *CDF5* induces cell elongation by upregulating growth- and cell wall-related genes (Martín et al. 2018).

CYCLING DOF FACTORS are a small sub-family within the plant specific DNA BINDING WITH ONE FINGER (DOF) transcription factor family, originally described as negative regulators of flowering time (Song et al. 2015). In contrast to *CDF5*, whose expression is shaped by the clock and the PIFs, *CDF1*, *CDF2*, *CDF3* and *CDF4* expression is almost exclusively regulated by the clock (Song et al. 2015). CDF transcriptional activity is directly correlated with specific post-translational regulation, which accounts for their differential accumulation under LD and SD (Song et al. 2015). The founding member *CDF1* was initially described as an inhibitor of photoperiod-dependent flowering time due to its circadian-dependent regulation provided by the F-box protein FKF1 (FLAVIN-BINDING, KELCH REPEAT, F-BOX 1) (Imaizumi et al. 2005). This regulation promotes *CDF1* ubiquitination and degradation, releasing the transcriptional repression of the *CDF1* direct targets *CO* and *FT*, which will promote flowering under LD photoperiods (Imaizumi et al. 2005, Song et al. 2012). Further work has shown that other CDF family members could also act as transcriptional repressors of *CO* and *FT* expression, and consequently inhibit flowering time (Fornara et al. 2009). Our results also confirmed *CDF5* role as a negative regulator of photoperiod dependent flowering. Moreover, we found that *CDF5* waveform is regulated by *FLORE* (*CDF5* LONG NON-CODING RNA), a natural antisense lncRNA transcript expressed antiphasic to *CDF5*, which promotes flowering (Henriques et al. 2017). These findings further confirmed the role of CDFs as molecular links connecting the circadian clock to photoperiod-dependent regulation of flowering time.

Here, we expand on our previous work by investigating the role of *CDF5* in the photoperiodic response of hypocotyl elongation. We found that *CDF5* deficiency leads to shorter hypocotyls in SD, but not in LD. Moreover, our results indicate that constitutively overexpressed *CDF5* is only able to promote growth under SD, and that *CDF5* is subjected to photoperiod dependent post-translational regulation. Furthermore, we describe a role for *CDF1* as promoter of hypocotyl elongation similar to *CDF5*, and show that this effect is also subjected to photoperiod-dependent post-translational regulation. Together, our findings suggest that tight regulation of *CDF5* accumulation and activity contributes to the photoperiodic control of hypocotyl elongation.

Materials and methods

Plant materials and seedling growth conditions

Arabidopsis thaliana seeds used in this study have been described elsewhere, including *CDF5OX* (*CDF5OX* 5.7 is used in main figures; Martín et al., 2018), *cdf5* (*cdf5-1*; Fornara et al., 2009), *cdf5-5'utr* (Henriques et al. 2017) and *pCDF1::HA-CDF1* (Imaizumi et al. 2005). The β -estradiol inducible *CDF5* line *pER8:Myc-CDF5* was generated by transforming *Arabidopsis* plants with a Myc-*CDF5*

fusion under the control of the *pER8* promoter. Briefly, the *CDF5* cDNA sequence was obtained using the cDNA synthesis kit SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) as described (Henriques et al. 2017). This fragment was then introduced into the pENTR™ Directional TOPO® Cloning kit (Invitrogen) to generate the ENTRY Gateway® clones, which were transferred to the destination vector *pER8-Myc-Gateway*, modified from the original inducible vector (Zuo et al. 2000) to generate the *pER8:Myc-CDF5* construct. Transformed T2 plants were analyzed by western blot to confirm Myc-CDF5 accumulation upon induction with 10 μM β-estradiol (Sigma). All lines are in the Columbia (Col-0) ecotype. Seeds were sterilized and plated on Murashige and Skoog medium without sucrose as described (Monte et al. 2003). Seedlings were stratified for 4 days at 4°C in darkness, and then placed in short days (8 h light + 16 h dark) (70 μmol m⁻² s⁻¹) or long days (16 h light + 8 h dark) (70 μmol m⁻² s⁻¹), using Master TL5 H0 39W/840 fluorescent lamps (Philips) as source of white light. Fluence rates were measured with a SpectraPen mini (PSI). For hypocotyl measurements, seedlings were arranged horizontally on a plate and photographed using a digital camera (Nikon D80). Hypocotyl measurements were done using ImageJ (National Institutes of Health). At least 25 seedlings were measured to calculate the mean and s.e.m. in at least two biological replicates. Estradiol treatments in Fig. 3 were performed by growing *pER8:Myc-CDF5* seedlings on plates containing 10 μM of β-estradiol in DMSO. Control samples were incubated only in DMSO for the same period of time.

Protein extraction and immunoblots

Protein extracts were prepared from SD-grown and LD-grown *pER8:Myc-CDF5* seedlings incubated for 72h with 10 μM of β-estradiol (or DMSO as control) and harvested after the third day of growth at ZT0, ZT0.5 and ZT1. Tissue samples were collected and frozen in liquid nitrogen, and protein extraction was done according to Kiba and Henriques (2016). Briefly, samples were manually ground under frozen conditions before resuspension in 2× SDS-loading buffer (1:1 v:v ratio). Samples were vortexed and centrifuged for 20 min at 10000 g at 4°C to remove cell debris. The supernatants were transferred to new tubes and 60 μg of each sample were loaded in a 10% SDS-PAGE gel. Proteins were then transferred to PVDF membrane (Merck Millipore) in Tris-HCl/Boric Acid buffer for 1h at 100V and membranes were blocked in 7.5% milk in TBST buffer for 2h. Immunodetection of Myc-CDF5 was performed using a rabbit anti-Myc polyclonal antibody (Sigma, USA) (1:1000 dilution). Peroxidase-linked anti rabbit secondary antibody (1:3000 dilution; Agrisera Antibodies™, Sweden) and an ECL chemiluminescence kit (Agrisera Antibodies™, Sweden) were used for detection of luminescence using LAS-4000 Image imaging system (Fujifilm). The membrane was stained with Coomassie blue as a loading control.

Gene expression analysis

Quantitative RT-PCR, RNA extraction, cDNA synthesis and qRT-PCR were done as described (Sentandreu et al. 2011). Briefly, 1 µg of total RNA extracted using either the RNeasy Plant Mini Kit (Qiagen) treated with DNase I (Ambion) according to the manufacturer's instructions or with Maxwell® RSC Plant RNA Kit (Promega). 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 2 µl was used for real-time PCR (Light Cycler 480; Roche) using SYBR Premix Ex Taq (Takara) and primers at a 300 nM concentration. Gene expression was measured in three independent biological replicates (with the exception of results shown in Figs 1C and 3A, with only one biological replicate), in at least three technical replicates for each biological sample. Primers used to analyse *CDF5* (AT1G69570) (EMP525 and EMP526 for *CDF5* cDNA and EMP528 and EMP772 for CDS region of *CDF5*) and normalize to *PP2A* (AT1G13320) (Shin et al. 2007) were as described previously (Martin et al., 2018). Primers used to analyse *FLA9* (AT1G03870), *AGP4* (AT5G10430) and *YUCCA8* (AT4G28720) were described previously (Rawat et al. 2009, Martín et al. 2018). Expression data in Figs 1B and 4B are from PHASER (<http://phaser.mocklerlab.org>), using the settings "Short Day" and "Long Day". *CDF5* data was obtained searching with "Array element name 259834_AT". Expression data shown in Fig. 4A was obtained from Martín et al. (2016).

Statistical analysis

Hypocotyl length in Figs 1A, 3B, 4C and S1 was analysed using Tukey post hoc multiple comparison test (GraphPad Prism7). Statistically significant differences were defined as those with a *P*-value < 0.05. Gene expression data were analysed using GraphPad Prism7 for statistically significant differences from their control. *P* values were determined by homoscedastic Student's t-test for data in Figures 1 C, 1D, 2 and 3A. Statistically significant differences were defined as those with a *P*-value <0.05. Significance level is indicated as **P* <0.05, ***P* <0.01 and ****P* <0.001.

Results

The photoperiodic response of hypocotyl elongation correlates with levels of *CDF5* expression

Our previous results proposed a model whereby at dawn in SD, PIFs induce expression of the elongation-promoting DOF-factor *CDF5* (Martín et al. 2018). To characterize whether *CDF5* might play a role in the photoperiodic response of hypocotyl elongation, we grew *CDF5*-deficient mutants in SD and LD. Two different alleles of *cdf5* [*cdf5-1* (Fornara et al. 2009) and *cdf5-5'utr* (Henriques et al. 2017)] displayed shorter seedling hypocotyl in SD compared to WT [in accordance to (Martín et al. 2016)]. In contrast, *cdf5* seedlings displayed normal hypocotyl elongation in LD (Figs 1A and S1). These results indicate that *CDF5* promotes hypocotyl elongation in SD but does not have a major role in this process in LD. Available expression data showed that the induction of *CDF5* expression that

occurs in the end of the night in SD is lacking in LD, where its expression waveform shows a clear reduction in amplitude mostly due to a very moderate induction at dawn (Fig. 1B, C). This is in agreement to the lesser accumulation of PIFs in LD conditions compared to SD (Nozue et al. 2007, Soy et al. 2012).

Constitutive *CDF5* expression promotes increased elongation in SD but not in LD

To further understand the role of *CDF5*, we investigated the phenotype of *CDF5* overexpressing seedlings (*CDF5OX*) in SD and LD. Overexpression of *CDF5* in this line is driven by the constitutive 35S promoter (Martín et al. 2018), and levels of *CDF5* transcript are comparable in SD and LD (Fig. 1D), ruling out any particular photoperiodic-specific post-transcriptional regulation that would differentially affect *CDF5* transcript levels under SD and LD conditions. As previously reported, *CDF5OX* exhibited enhanced hypocotyl elongation in SD (Martín et al. 2018). Remarkably, however, this phenotype was absent in LD-grown *CDF5OX* seedlings (Figs 1A and S1). Correlated with the photoperiodic-specific elongation activity, expression of the growth and cell wall genes *YUC8*, *AGP4* and *FLA9* was promoted in *CDF5OX* seedlings under SDs, in agreement with our previous findings (Martín et al. 2018). However, their expression was not affected compared to the WT control under LD (Fig. 2). These results indicate that in LD, *CDF5* is subjected to LD-specific post-transcriptional regulation affecting its activity.

Together, these data suggest that plants minimize PIF-regulated, *CDF5*-mediated growth under LD by at least two LD-specific mechanisms: (1) by keeping the levels of *CDF5* expression low (Fig. 1C, D), and (2) by subjecting *CDF5* to differential post-transcriptional regulation in SD and LD (Figs 1A, E, 2 and S1).

***CDF5* activity in promoting cell elongation is photoperiod regulated**

To further explore the photoperiod-specific post-transcriptional regulation of *CDF5*, we made use of an inducible *pER8::Myc-CDF5* line carrying a N-terminal Myc tag. Induction in SD and LD using β -estradiol resulted in similar levels of *CDF5* transcript in both photoperiodic conditions (Fig. 3A), further confirming the absence of any photoperiod-specific mechanism inhibiting *CDF5* transcript accumulation. We then checked *CDF5* protein levels at ZT0, ZT0.5 and ZT1, dawn time points that span the maximum growth rate window under SD (Nozue et al. 2007). Notably, we detected accumulation of *CDF5* in both SD and LD (Fig. 3B). Whereas the protein levels were similar in all three time points within each photoperiod, *CDF5* protein levels appeared higher under LD when compared to SD. However, despite the accumulation under LDs, there was no growth promotion in these lines, in clear contrast to SD (Fig. 3C). These results indicate that *CDF5* protein in LDs, even if accumulated to higher levels than SD, is not active in the promotion of hypocotyl elongation, and

suggest a tight photoperiodic-specific post-translational regulation of CDF5 that could modulate its transcriptional activity.

Photoperiod-specific regulation of hypocotyl elongation extends to other CDF-family members

In adult *Arabidopsis* plants, CDF5 is a regulator of photoperiodic flowering together with other DOF transcription factors (Imaizumi et al. 2005, Fornara et al. 2009). Interestingly, *CDF5* is the only member of the CDF clade (which includes *CDF1*, *CDF2*, *CDF3*, *CDF4*, *CDF5*, and *COG1*) under direct control of the PRRs and PIFs based on available data in young seedlings (Fig. 4A) (Pfeiffer et al. 2014, Liu et al. 2016). In accordance, the pattern of expression in SD and LL (entrained in SD and then released in continuous light) for *CDF5* is unique, with high levels at dawn in SD that are PIF-dependent, and low levels in LL (Martín et al. 2016) (Fig. 4A). In contrast, expression of *CDF1-CDF4* and *COG1* are mainly regulated by the clock (Imaizumi et al. 2005, Fornara et al. 2009). Importantly, the protein levels of at least CDF1 are tightly controlled, accumulating only at dawn. The proposed mechanism for the regulation of flowering involves the repression of *CONSTANTS (CO)* expression by CDFs in the morning hours in SD and LD, restricting accumulation of CO to the late hours of long days due to FKF1-mediated degradation of CDFs, and consequent accumulation of *FLOWERING LOCUS T (FT)* to promote flowering (Imaizumi et al. 2005). In accordance, a *cdfq* mutant deficient in *CDF1*, 2, 3 and 5 is photoperiod-insensitive and early flowering (Fornara et al. 2009). Because accumulation of CDF1 was reported to coincide with dawn (Fig. 4B) (Imaizumi et al. 2005), we wondered whether CDF1 could promote growth in young seedlings similarly to CDF5. Interestingly, a line expressing *CDF1* under the control of its own promoter (*pCDF1::HA-CDF1*) to levels ~5× higher than WT (Imaizumi et al. 2005), promoted hypocotyl elongation in SD but was not as active as in LD (Fig. 4C), even though CDF1 protein accumulated for longer (ZT1-ZT7) in this photoperiod (Imaizumi et al. 2005). These results are similar to our findings for CDF5. They indicate that CDF1 has the capacity to promote hypocotyl cell elongation similarly to CDF5, and that this activity is photoperiod-specific and restricted to SD. Together, these data hint towards a post-translational mechanism acting in LD to inhibit CDF5 activity in hypocotyl cell elongation that might be common for CDF1.

Discussion

We expand here on our previous identification of *CDF5*, a DOF transcription factor, as a direct co-target of the sequential morning-to-midnight repression of PIF function by waves of PRRs, which ensures the gating of hypocotyl growth to dawn under SD conditions (Martín et al. 2018). Under LDs, on the other hand, hypocotyl elongation is much reduced in a PIF-dependent manner (Niwa et al. 2009), and the *pifq* mutant is relatively insensitive to photoperiod (Gommers and Monte 2018). Our findings lead us to propose a model whereby photoperiodic regulation of hypocotyl elongation is underlain by (1) PIF-mediated transcriptional induction of growth enhancers such as *CDF5*,

specifically under SDs which coincide with greater accumulation of PIFs, and (2) photoperiod-specific post-translational regulation that modulates CDF1 and CDF5 transcriptional activity under LD.

Photoperiod specific CDF5 promotion of hypocotyl elongation

Our results describe CDF5 as a relevant actor in the photoperiodic control of hypocotyl elongation, and revealed a level of photoperiodic-specific post-translational regulation previously unrecognized. This novel function in cell elongation could be explained solely by the differential expression waveform and amplitude of *CDF5* expression (and other genes involved in the promotion of hypocotyl cell elongation) between SD and LD (Fig. 1C, D), which could be due to both clock- and PIF-regulated transcription. However, our results showing that constitutive expression of *CDF5* in *CDF5OX* failed to promote gene expression and hypocotyl elongation in LD despite accumulating similar transcript levels to SD (Figs 1 and 2), strongly suggested additional layers of post-transcriptional regulation. Indeed, our data showed that although Myc-CDF5 accumulation in *pER8* inducible lines was similar or even higher in LD-grown seedlings, it could only promote elongation under SD conditions, indicating that photoperiod-dependent post-translational modifications modulating CDF5 transcriptional activity are likely in place in LD. This multi-level regulation is reminiscent of other photoperiodic specific responses. In Arabidopsis, flowering induction takes place under LD and is repressed in SD by members of the CDF family (Song et al. 2015), which are subjected to photoperiod-specific post-translational mechanisms gating CDF protein accumulation and action. CDF1 total protein levels were previously shown to be under photoperiod control, accumulating preferably from ZT1-ZT4 under SD and ZT1-ZT7 under LD photoperiods regardless of promoter tested (e.g. CMV 35S or *CDF1* own promoter) (Imaizumi et al. 2005). Under SD, CDF1 represses *CO* expression during the day hours preventing *FT* accumulation and flowering. Under LD, CO accumulates during the second half of the day inducing *FT* expression and flowering (Imaizumi et al. 2005).

Photoperiod regulates CDF1 and CDF5 protein accumulation and hypocotyl elongation activity

Interestingly, although *CDF5* regulation by PIFs appears to be unique among the CDF-clade members (Fig. 4A), *CDF1* expression in seedlings displayed a similar oscillatory pattern to *CDF5* under SD (Fig. 4B). Its transcript levels start to rise at ZT20, and peak at ZT0, with slightly higher levels in SD (Fig. 4B). This oscillatory behavior suggested that CDF1 might also play a role in photoperiodic regulation of hypocotyl elongation. Indeed, using previously described *pCDF1::HA-CDF1* lines where a *CDF1* minigene is expressed under the native *CDF1* promoter to levels ~5-fold those of the endogenous *CDF1* at dawn in both SD and LD (Imaizumi et al. 2005), we could show that CDF1 in SD can clearly promote hypocotyl elongation (Fig. 4C). However, similarly to CDF5, hypocotyl elongation was only marginally promoted under LD (Fig. 4C), although under these conditions, and due to its main regulation by the clock, *CDF1* expression is also rhythmic and with an amplitude similar to SD, peaking at ZT0-ZT4 (Imaizumi et al. 2005) (Fig. 4B). These findings further strengthen

our hypothesis that CDF5 transcriptional activity, and likely CDF1, specifically promotes the expression of growth-inducing genes under SD photoperiods, and this regulation seems to be absent in LD-grown seedlings. This differential photoperiodic behavior could be due to the combination of reduced accumulation of *CDF* under LDs (especially for *CDF5*) and photoperiod-dependent post-translational modifications, which could modulate protein activity and/or the ability to form active complexes. Another possibility that cannot be discarded based on our data is photoperiod-specific availability of putative obligate partners for CDF activity.

CDF transcription factors have widespread biological functions

CDFs belong to the wider family of plant specific DOF transcription factors, which are involved in different aspects of plant life from germination (Boccaccini et al. 2016), to vascular differentiation and root radial growth (Miyashima et al. 2019, Smet et al. 2019), abiotic stress responses (Corrales et al. 2014, Corrales et al. 2017), [regulation of tuberization](#) (Kloosterman et al. 2013) and flowering time regulation (Fornara et al. 2009). *DOF AFFECTING GERMINATION 1 (DAG1)* has been implicated in hypocotyl elongation in seedlings grown under continuous red light, possibly due to transcriptional regulation of several ABA, ethylene and auxin related genes (Lorrai et al. 2018). Within the DOF family, CDFs have mostly been known for their role as negative regulators of flowering time (Song et al. 2015). Recently, however, CDFs have also been associated with the regulation of abiotic stress responses both in *Arabidopsis* and tomato (Corrales et al. 2014, Corrales et al. 2017). Moreover, the GI-CDF module was implicated in hypocotyl growth regulation in older seedlings, although the mechanism for this regulation was not investigated (Fornara et al. 2015). [Interestingly, CDF1 is also involved in a temporal response to nitrogen especially in shoots, suggesting a role in nutrient response](#) (Varala et al. 2018). Our findings here expand on these functions by showing that CDFs are involved in the early stages of photomorphogenesis by promoting hypocotyl elongation in shorter photoperiods. We also show that this function is distinct from their role in flowering time regulation. Whereas hypocotyl elongation requires accumulation at dawn preceded by a long night, flowering regulation directly correlates with CDF accumulation throughout the light period (Song et al. 2015). Most likely this functional diversity will correlate with specific transcriptional target activity possibly due to CDF participation in multiple protein complexes. Our results support this possibility, since CDF1 and CDF5 act as positive regulators of hypocotyl elongation most likely by promoting the expression of cell elongation genes, similarly to their role in abiotic stress responses (Corrales et al. 2014, Corrales et al. 2017) and in contrast to their role as repressors of gene expression in the regulation of flowering time. Together these findings highlight the functional diversity of these transcriptional regulators during different stages of *Arabidopsis* development and in response to a diversity of environmental signals.

Author contributions

G.M., N.V., R.H. and E.M. designed the work and analyzed data. G.M., N.V., M.B. and A.R. acquired the data. G.M., N.V., R.H. and E.M. wrote the manuscript. All authors revised and approved the manuscript's content.

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Data availability statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Supporting information

Fig. S1. Hypocotyl length of WT, *cdf5*, and *CDF5OX* (lines 5.7, 1.8 and 10.9) grown for 3 or 4 days in SD (top) or LD (bottom). Data are means \pm SEM of at least 35 seedlings. Different letters denote statistically significant differences among means by Tukey test ($P < 0.05$) relative to its respective WT.

Figure Legends

Fig. 1. (A) (Left) Hypocotyl length of WT, *cdf5*, *cdf5-5'UTR* and *CDF5OX* grown for 3 days in SD (left) or LD (right). Data are means \pm SEM of at least 25 seedlings. Different letters denote statistically significant differences among means by Tukey test ($P < 0.05$). (Right) Visible phenotypes of 3-day-old seedlings grown in SD (left) and LD (right). Scale bar =5 mm. (B) Comparison of *CDF5* expression in SD and LD conditions. *CDF5* expression data was obtained from the publicly available DIURNAL website (<http://diurnal.mocklerlab.org/>). (C) *CDF5* expression at ZT0 and ZT1 in SD (left) and LD (right) of WT seedlings grown for three days in SD or LD and harvested at ZT0 or ZT1. Data are from three independent technical replicates and are relative to WT SD ZT0 *CDF5* expression set as one. Error bars indicate SEM. (D) *CDF5* expression in WT, *cdf5* and *CDF5OX*. Seedlings were grown in SD and LD and harvested at ZT0. Data are from three independent biological replicates and relative to WT SD set at one. Error bars indicate SEM.

In C and D, *CDF5* expression was analysed by qRT-PCR and normalized to *PP2A*. Statistically significant differences between mean values by Student's t-test relative to WT SD ZT0 and WT in each photoperiod, respectively, are shown (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). n.s., not significant.

Fig. 2 . Expression of PIF-regulated growth (*YUCCA8*) and cell wall (*AGP4*, *FLA9*) marker genes in 3-day old SD (left) and LD (right) WT, *cdf5* and *CDF5OX* seedlings at ZT0, analysed by qRT-PCR and normalised to *PP2A*. Data are from three independent biological replicates and relative to WT SD *CDF5* expression value set as one. Error bars indicate SEM. Statistically significant differences between mean values by Student's t-test relative to WT in its own condition are shown (* $P < 0.05$, ** $P < 0.001$). n.s., not significant.

Fig. 3. (A) *CDF5* expression in *pER8::Myc-CDF5* seedlings grown under SD or LD conditions for 3 days in medium with (+) or without (-) β -estradiol. Samples were taken during the following day at specified time points. Data was analysed by qRT-PCR and normalised to *PP2A*. Data are from three

independent technical replicates and relative to WT SD ZT0 (- β E) β -estradiol *CDF5* expression set as one. Statistically significant differences between mean values by Student's t-test relative to WT in each condition are shown ($***P < 0.001$). n.s., not significant. (B) Immunoblot of protein extracts from *pER8::Myc-CDF5* seedlings grown in β -estradiol-containing media. Seedlings were grown under SD or LD conditions for 3 days and samples were taken during the following day at specified time points. Anti-Myc antibody was used to detect MYC-CDF5 protein. Coomassie blue staining was used as loading control. (C) (Top) Hypocotyl length of inducible *pER8::Myc-CDF5* lines grown in SD and LD conditions in media with (+ β E) or without (- β E) β -estradiol. (Bottom) Visible phenotypes of 3-day-old seedlings grown with (+) or without (-) β -estradiol. Scale bar = 5 mm.

Fig. 4. (A) Expression of six *CDF5* clade members in 3d-old WT and *pifq* seedlings at dawn. Seedlings were grown for 2 d in SD conditions and samples were harvested during the third day in seedlings kept in SD at ZT0 (SD) or transferred to free running conditions at CT0 (LL). Bar graphs of microarray data (Martín et al. 2016) show the fold change in gene expression relative to the WT SD at ZT0. Data correspond to biological triplicates, and bars indicate SEM. Binding of PIF1/3/4/5 and PRR5/7/9 is indicated with filled squares on top of each graph, based on data from Pfeiffer et al. (2014) and Liu et al. (2016). Statistically significant differences between mean values by Student's t-test relative to WT in its own condition are shown ($*P < 0.05$; $**P < 0.001$ and $***P < 0.001$). n.s., not significant. (B) Comparison of *CDF1* expression in SD and LD conditions. *CDF1* expression data was obtained from the publicly available DIURNAL website (<http://diurnal.mocklerlab.org/>). (C) (Left) Hypocotyl length of WT and *pCDF1::HA-CDF1* seedlings grown for 3 days in SD or LD. Data are means \pm SEM of at least 40 seedlings. Different letters denote statistically significant differences among means by Tukey test ($P < 0.05$) relative to WT SD. (Right) Visible phenotypes of 3-day-old seedlings grown in SD and LD. Scale bar =5 mm.

A







