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Gibberellin driven growth in *elf3* mutants requires PIF4 and PIF5

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Abbreviations: CO, CONSTANS; Col-0, Columbia; EC, Evening Complex; ELF3, EARLY FLOWERING 3; ELF4, EARLY FLOWERING 4; EMS, ethyl methanesulfonate; FT, FLOWERING LOCUS T; GA, gibberellin; GA20ox, gibberellin 20-oxidase; GA3ox, gibberellin 3-oxidase; LD, long day; LUX, LUX ARRHYTHMO; PAC, paclobutrazol; PIF - PHYTOCHROME INTERACTING FACTOR; qPCR, quantitative RT-PCR; SD, short day; WT, wild type; ZT, Zeitgeiber Time.

The regulatory connections between the circadian clock and hormone signaling are essential to understand, as these two regulatory processes work together to time growth processes relative to predictable environmental events. Gibberellins (GAs) are phytohormones that control many growth processes throughout all stages of the plant life cycle, including germination and flowering. An increasing number of examples demonstrate that the circadian clock directly influences GA biosynthesis and signaling. EARLY FLOWERING 3 (ELF3) participates in a tripartite transcriptional complex known as the Evening Complex (EC). In this capacity, ELF3 is fundamental to core circadian clock activity, as well as time-of-day specific regulation of genes directly responsible for growth control, namely the *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) and *PIF5* genes. Here we show that the GA biosynthesis inhibitor paclobutrazol substantially reduces the long hypocotyl and petiole phenotypes of Arabidopsis *elf3* mutants. In addition, loss of ELF3 activity causes upregulation of the key GA biosynthesis genes *GA20ox1* and *GA20ox2*. Moreover, *GA20ox1* and *GA20ox2* expression depends strongly on the redundant activities of *PIF4* and *PIF5*. These findings indicate that the defining growth phenotypes of *elf3* mutants arise from altered GA biosynthesis due to misregulation of *PIF4* and *PIF5*. These observations agree with recent work linking increased GA production with the elongated growth phenotypes of the barley *elf3* mutant. Thus, the role of the EC in regulation of GA biosynthesis and signaling in eudicots is shared with monocots and, therefore, is a highly conserved mechanism for growth control.

Introduction

Plants must coordinate growth processes with the predictable daily environmental changes accompanying daytime and nighttime.[1,2] The plant circadian clock accomplishes this by imparting rhythmic patterns with approximately 24-hour periods to biological processes and their underlying genes, which confines these processes to defined times within the day.[3-6] The plant circadian clock is set (or entrained) by light and temperature cues, while a core molecular oscillator comprised of transcription-translation feedback loops sustains rhythmic activity.[6,7] The molecular interactions within the oscillator compose of a series of primarily repressive regulatory events, which involve numerous proteins that themselves have peak abundances and activities at specific times within the day.[8,9] Progress in the last two decades has significantly advanced the understanding of how the core clock functions; however, many mechanistic connections between the core molecular oscillator and physiological outputs remain incompletely understood.

A fundamental role of the circadian clock is to communicate timing information to diverse "output" processes, including growth, development, and metabolism (for reviews, see refs. 10-12). An example of direct coupling between the core oscillator and growth outputs involves the activity of the *Arabidopsis thaliana* evening complex (EC).[13,14] The tripartite EC protein complex directly represses daytime-phased genes and allows clock progression from day to night.[13-16] At least 3 different dusk-expressed proteins compose the EC: LUX ARRHYTHMO/PHYTOCLOCK 1 (LUX/PHY1), EARLY FLOWERING 4 (ELF4), and EARLY FLOWERING 3 (ELF3).[13,17-19] LUX is a MYB-like GARP transcription factor that targets the EC to promoters by sequence-specific binding of *cis* elements upstream of many day-expressed genes.[14,18-20] Recruitment of the EC to promoters also requires NOX/BOA, a MYB transcription factor in the same family as LUX.[14,20,21] The exact biochemical functions of both ELF3 and ELF4 are presently unclear, as neither protein has recognizable functional domains; however, both are required

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for EC activity since daytime-phased genes are highly expressed throughout the night in *elf3* mutant Arabidopsis seedlings.[15,16,22,23]

In addition to control of core clock genes, the EC regulates expression of genes encoding important components of growth control networks, including the *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) and *PIF5* genes.[13,14] PIF4 and PIF5, along with other members of the PIF transcription factor family, are central regulatory hubs where multiple internal and external cues converge and are integrated to control diverse plant growth processes, including germination, skotomorphogenesis, chloroplast differentiation, shade-avoidance, and flowering (for review, see ref. 24). PIF protein abundance and activity is defined by a combination of circadian clock-directed gene expression and post-translational mechanisms that detect light, temperature, and phytohormone presence.[25-28] Together, these regulatory mechanisms tightly constrain the bulk of PIF4 and PIF5 activity in young seedlings to a short time period just prior to dawn.[25,29] The circadian clock generates rhythmic *PIF4* and *PIF5* expression through EC-imposed repression at dusk that subsequently declines over the pre-dawn hours as EC activity feeds back to inhibit expression of *LUX* and *ELF4*.[13,20] As a result, *PIF4* and *PIF5* gene expression increases in the hours before dawn and continues throughout the day so that *PIF4* and *PIF5* transcripts have peak expression at approximately midday.[25,30] However, PIF4 and PIF5 protein accumulation is maximal only immediately before dawn because accumulated PIF proteins are rapidly degraded in the first light of day by a phytochrome B- and ubiquitination-dependent pathway.[25,26,31,32]

PIF4 and PIF5, along with PIF1 and PIF3, contribute to elongation growth in dark-grown seedlings.[25,29,33] However, PIF4 and PIF5 mainly drive elongation growth in green seedlings. An important activity of these transcription factors is to positively regulate hypocotyl elongation in pre-dawn hours under diurnal conditions.[25,34,35] The typical elongated hypocotyl phenotype of arrhythmic Arabidopsis clock mutants, like *elf3*, *elf4*, and *lux*, is largely due to high, nearly constitutive *PIF4* and *PIF5* expression.[13,25] Continuous gene expression causes greater PIF protein accumulation throughout the entire night and consequent growth enhancement, rather than the normal restriction of growth to pre-dawn hours.[13,25] As a result, *elf3-2 pif4-101* and *elf3-2 pif5-1* double mutants have shorter hypocotyls relative to *elf3-2* seedlings, and the *elf3-2 pif4-101 pif5-1* triple mutant has a hypocotyl length indistinguishable from wild type (WT) seedlings.[13]

PIF transcription factors promote growth by binding to G-box and E-box variant *cis*-elements in the promoters of target genes, including genes involved in phytohormone biosynthesis and signaling.[35,36] PIF1, PIF3, PIF4, and PIF5 together regulate thousands of genes, including many genes involved in hormone biosynthesis and signaling.[36-38] PIF4, PIF5, and PIF7 activate expression of *TAA1, CYP79B2*, *YUC8*, and *YUC9* genes, which encode enzymes required for the rate-limiting steps of distinct auxin biosynthetic pathways.[35,39,40]

GA biosynthesis is highly regulated, primarily though transcriptional control of *gibberellin 20-oxidase* (*GA20ox*) and *gibberellin 3-oxidase* (*GA3ox*) genes, which encode enzymes for catalysis of the final reactions in the production of bioactive GA_1 and GA_4.[41] In a series of oxidation steps, GA20ox enzymes convert GA_{12} and GA_{53} to GA_9 and GA_{20}, respectively. GA3ox enzymes then produce GA_1 and GA_4 from these substrates. Of the 5 paralogous *GA20ox* genes in the Arabidopsis genome, *GA20ox1* and *GA20ox2* are primarily required for GA biosynthesis, while *GA20ox3* contributes to a lesser degree.[42,43] *GA20ox1* is most important, as loss of it alone reduces internode elongation, while single mutants of the others do not affect growth.[42] The contribution of *GA20ox2* is revealed only when combined with a *ga20ox1* mutant. Similarly, loss of *GA20ox3* exacerbates the phenotypes of a *ga20ox1* mutant.[43] At present, the transcriptional networks that regulate *GA20ox* and *GA3ox* expression in Arabidopsis are incompletely understood. PIF1/PIL5 represses *GA3ox1* and *GA3ox2*.[37,44] PIF1/PIL5 also binds to *GAI* and *RGA* promoters, which encode repressors of GA signaling, and promotes expression of these genes, but apparently does not bind to the promoters of other GA biosynthetic genes, including *GA3ox1*, *GA3ox2*, *GA2ox2*, and any of the *GA20ox* paralogs.[44]

While the circadian clock influences *PIF* gene expression, and PIF proteins impact hormone signaling, how these components work together as a module is unclear. For example, mutants of Arabidopsis *elf3*, which by definition are mutants for EC activity, are well-studied and characterized with respect to their effect on core circadian clock function, but the impact of losing ELF3 activity (i.e., EC function) on growth regulatory pathways is less defined, aside from a clear role in direct regulation of *PIF4* and *PIF5* expression.[13,22] The Arabidopsis *elf3-1* allele, like all *elf3* null mutants, has pale green leaves and cotyledons, in addition to elongated hypocotyl and petiole growth.[13,45-47] This constellation of phenotypes resembles plants with an increased GA response.[48,49] The putative GA hyper-response phenotype in *elf3* is especially pronounced when plants are grown in short day (SD; 8 hours light/16 hours dark) photoperiods.[45] GA biosynthesis and signaling is deeply entwined with the circadian clock. The daily expression pattern of many GA metabolism and signaling genes is rhythmic.[5] GA sensitivity and responses also are limited to discrete times of day by the action of the circadian clock.[5,50-53] Additionally, the growth phenotypes of a barley *elf3* mutant, which include elongated coleoptiles and extended pale-green leaves, result from greater GA abundance.[54] Here, we show that elongated growth phenotypes in Arabidopsis *elf3* mutants are GA-dependent. In addition, we demonstrate an important regulatory connection between the EC and GA-dependent growth that involves the growth-promotion activity of PIF4 and PIF5.

Results

GA biosynthesis contributes to the *elf3-1* phenotype

The Arabidopsis null *elf3-1* mutant was tested for GA biosynthesis-dependent phenotypes by assessing the consequences of paclobutrazol (PAC) treatment on hypocotyl elongation in WT and *elf3-1* seedlings. PAC inhibits an initial enzymatic step in GA biosynthesis, which is upstream of the action of GA20ox and

GA3ox enzymes.[55] As expected, untreated *elf3-1* seedlings have severely elongated hypocotyls that are nearly 3 times longer than WT under SD conditions (**Fig. 1A and B**). Five days of PAC treatment significantly diminishes the hyper-elongation phenotype in *elf3-1* seedlings and the strength of the effect is dependent on the PAC concentration. Increasing the PAC concentrations from 0.1 to 10 μM progressively suppresses hypocotyl elongation in *elf3-1* seedlings to the point where the difference between WT and *elf3-1* is only 30% at 1 and 10 μM PAC (**Fig. 1A and B**). The strong inhibitory effect of PAC on *elf3-1* hypocotyl elongation indicates that the majority of this growth is due to enhanced GA biosynthesis. Notably, the difference between WT and *elf3-1* seedlings at both 1 and 10 μM PAC remains significant (unpaired t-test, $p < 0.05$), which shows that additional factors beyond GA make a small contribution to hypocotyl growth in the *elf3* mutant.

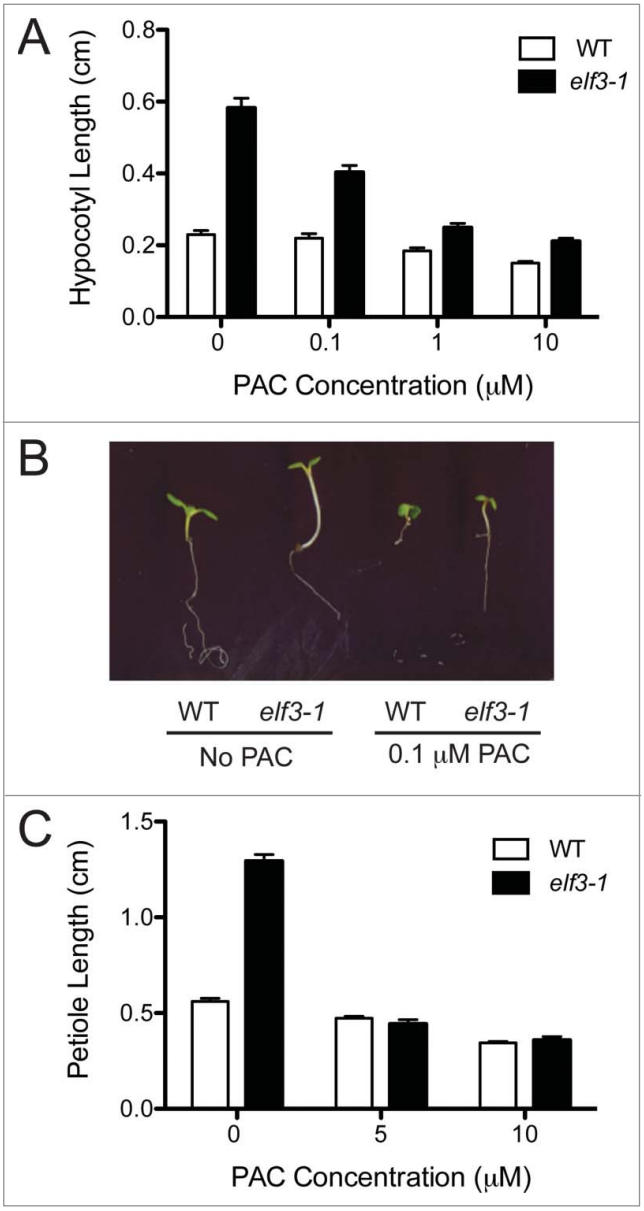

Figure 1. Inhibition of GA biosynthesis reduces elongated growth in the *elf3-1* mutant. (**A**) Hypocotyl lengths of 7 day-old WT (open bars) and *elf3-1* (black bars) seedlings grown under SD on the indicated concentrations of GA biosynthesis inhibitor PAC for the last 5 d before measurement. Shown is the mean (±SEM) from 3 independent experimental replicates of n = 20 seedlings each. (**B**) Seven day-old WT and *elf3-1* seedlings grown in SD without PAC or with 0.1 μM PAC grown as indicated. (**C**) Mean (±SEM) petiole lengths of 28 day-old WT and *elf3-1* plants exposed to the indicated PAC concentration for 3 weeks. Measurements are from a single experimental replicate (n = 15) grown in SD.

PAC treatment of older plants confirms that other growth phenotypes of *elf3-1* are also GA biosynthesis-dependent. The petiole length of 28 day-old *elf3-1* plants is more than twice that of age-matched WT plants (**Fig. 1C**). On the other hand, petiole length of the two genotypes is indistinguishable in the presence of either 5 or 10 μM PAC (**Fig. 1C**). Taken together, these observations show that GA biosynthesis is a major contributing factor to the Arabidopsis *elf3-1* elongation growth phenotype.

GA biosynthesis genes are highly expressed in *elf3* mutants

The sensitivity of hypocotyl and petiole elongation to PAC in *elf3-1* indicates that the pronounced growth of these two organs in the mutant arises from higher than normal GA production. Since the *elf3-1* mutant causes large-scale changes in transcription for circadian clock-regulated genes, a potential mechanism for enhanced GA production in the *elf3-1* background is through misregulation of genes required for GA biosynthesis.

Expression of *GA20ox1* and *GA20ox2* exhibits a rhythmic pattern in WT with a time of peak expression that depends on the photoperiod conditions. In SD conditions, the transcript of each rises throughout the night and peaks at dawn (**Fig. S1A and B**). Interestingly, the pattern for each gene is changed in long day (LD; 16 hours light/8 hours darkness) photoperiods: expression begins to rise at dawn and the time of maximal expression is shifted 8 hours into the day (**Fig. S1A and B**). Thus, *GA20ox1* and *GA20ox2* expression is adjusted according to photoperiod, which is consistent with the circadian clock contributing to the expression behavior of these genes.

Loss of *ELF3* activity causes strong upregulation of both *GA20ox1* and *GA20ox2* expression in SD conditions, but has little effect on the expression of the other *GA20ox* paralogs. Quantitative RT-PCR (qPCR) analysis of *GA20ox1* expression at dawn (Zeitgeiber Time 0 (ZT0) hours) in two different *elf3* mutant alleles, *elf3-1* and *elf3-2*, reveals significantly higher transcript levels in each mutant background compared to WT seedlings (unpaired t-test, $p < 0.05$ and $p < 0.0005$, respectively) (**Fig. 2A**). *GA20ox2* is also significantly induced in *elf3-1* and *elf3-2* to nearly the same degree as *GA20ox1* (unpaired t-test, $p < 0.01$ and $p < 0.05$) (**Fig. 2B**). On the other hand, neither mutant allele changes the expression behavior of *GA20ox3*, *GA20ox4*, or *GA20ox5* to any significant degree (**Fig. 2C–E**). *GA3ox1* encodes the enzyme acting immediately downstream of GA20ox that is responsible for producing bioactive GAs. Expression of this gene is also not changed by either mutant (**Fig. 2F**). Therefore, ELF3 is required to repress expression of both *GA20ox1* and *GA20ox2*, which indicates that EC activity normally suppresses these genes.

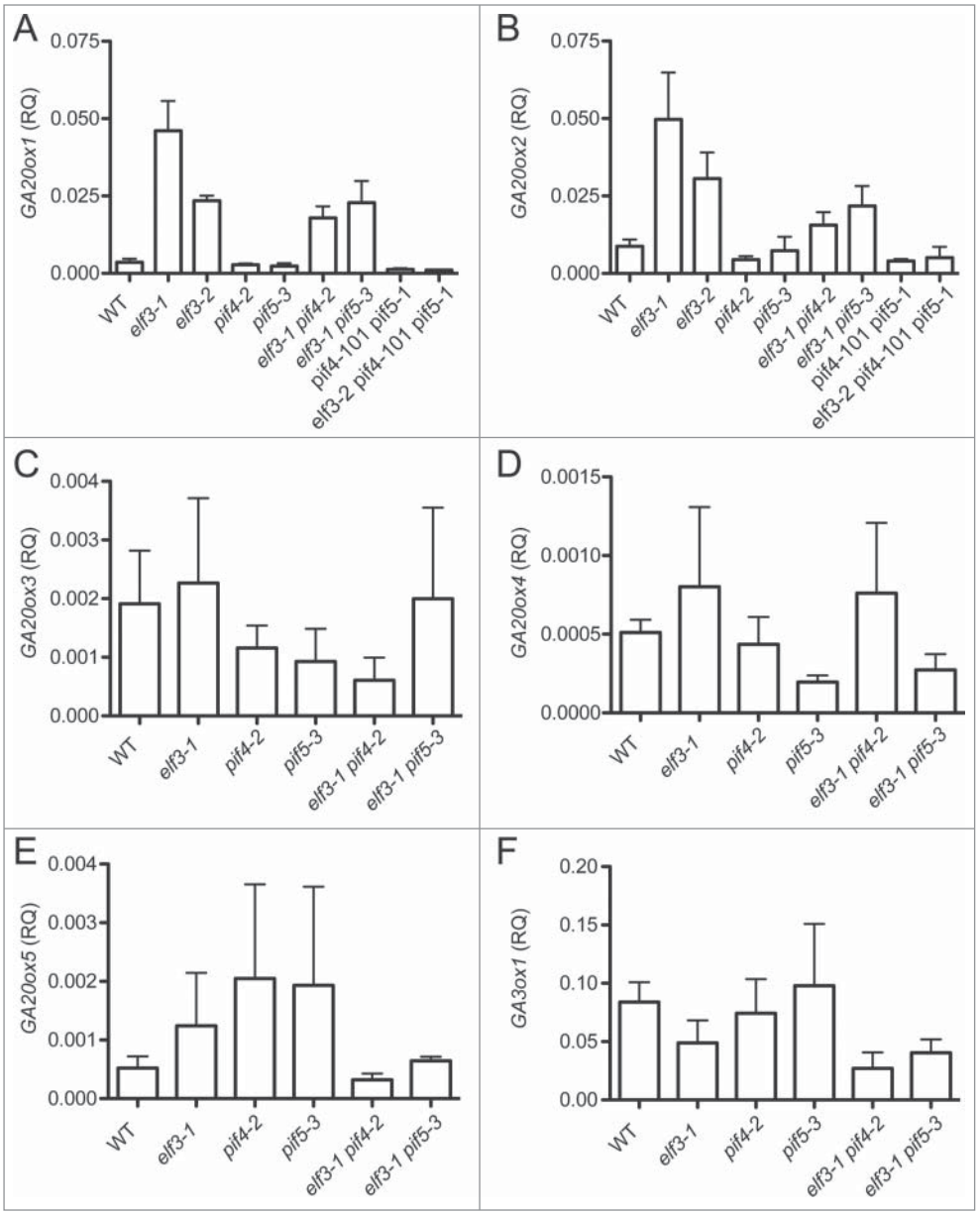

Figure 2. Elevated pre-dawn expression of GA biosynthetic genes in *elf3* mutants requires *PIF4* and *PIF5*. Transcript abundance for (**A**) *GA20ox1*, (**B**) *GA20ox2*, (**C**) *GA20ox3*, (**D**) *GA20ox4*, (**E**) *GA20ox5* and (**F**) *GA3ox1* in the indicated genotypes at 15 minutes before ZT0 (lights-on) under SD conditions. Transcript levels were measured by qPCR analysis. Genotypes are depicted on x-axis and y-axis displays relative quantitation (RQ) of transcript normalized to *PDF2* according to Materials and Methods. Shown is the mean (±SEM) of 3 independent experimental replicates.

GA20ox expression requires the transcription factors PIF4 and PIF5

To identify factors regulating growth downstream of *elf3-1* and the EC, a suppressor screen was carried out where ethyl methanesulfonate (EMS) mutagenized *elf3-1* plants grown in SD conditions were scored for restoration of WT-like growth habit. One suppressor line (*s41*) exhibited hypocotyl, petiole, and leaf shape that resembled WT plants (**Fig. S2A and B**). Mapping of the *s41* suppressor mutation identified a C to T transition in the *PIF4* gene that converts an arginine at position 182 to a premature stop codon (**Fig. S2C and D**). To confirm mutation of *pif4* was responsible for the suppressor phenotype and to further study the genetic interaction between the *ELF3* and *PIF4* genes, *elf3-1* was crossed to the *pif4-2* reference allele. Like the previously described *elf3-2 pif4-101* mutant of Nusinow et al.,[13] the *elf3-1 pif4-2* double mutant and the *s41* suppressor have comparable phenotypes, including reduced hypocotyl elongation, shorter petioles, and WT-like leaf shape.

Since the characteristic growth phenotypes of *elf3-1* and *elf3-2* both coincide with elevated *GA20ox* expression and depend on PIF4 and PIF5 activity, we tested whether either PIF4 or PIF5 influence *GA20ox* gene expression in WT and *elf3* mutant backgrounds. Combining either the *pif4-2* or *pif5-3* mutant with the *elf3-1* allele reduces the high *GA20ox1* and *GA20ox2* transcript levels of the single *elf3-1* mutant by 30% and 50% for *elf3-1 pif4-2* and *elf3-1 pif5-3*, respectively (**Fig. 2A and B**). Although the difference is apparent by eye, statistical testing shows only the changes for *GA20ox2* are statistically significant between the two populations (unpaired t-test, $p < 0.05$). In contrast, the single *pif4-2* and *pif5-3* alleles alone cause little change in *GA20ox1* and *GA20ox2* expression. Importantly, *GA20ox1* and *GA20ox2* transcript accumulation in *elf3-2 pif4-101 pif5-1* triple mutant seedlings is strongly reduced relative to the *elf3-2* parent (unpaired t-test, $p < 0.0005$ and $p < 0.05$, respectively) and the levels of each are near that of WT seedlings (**Fig. 2A and B**). Similar analysis for *GA20ox3*, *GA200ox4*, *GA20ox5*, and *GA3ox1* reveals no significant effect on expression caused by *pif4* and *pif5* mutants (**Fig. 2C–F**).

Like *GA20ox1* and *GA20ox2*, *PIF4* and *PIF5* are expressed in a rhythmic fashion.[25,56] A comparison of the expression profiles for *PIF4*, *PIF5*, *GA20ox1* and *GA20ox2* reveals striking similarities between them (**Fig. S2**). For all, the timing of peak expression is determined by photoperiod. In SD conditions, expression of all 4 genes begins after dusk and continues to rise until dawn, at which point expression drops for each, aside from *PIF4*.

Compared to the pattern in SD conditions, LD photoperiods cause a pronounced shift in peak expression into the day. The tight correlation in expression between the *PIF* transcription factor genes and the *GA20ox* genes is consistent with PIF4 and PIF5 playing a role in regulation of these GA biosynthesis genes. All together, the findings here indicate that EC influences GA biosynthesis through control of *GA20ox1* and *GA20ox2* expression in a pathway that requires the redundant action of PIF4 and PIF5.

Auxin biosynthesis and signaling genes are highly expressed in *elf3*, which requires PIF4 and PIF5 activity

The phytohormone auxin is an established regulator of GA biosynthesis for several plant processes, including hypocotyl elongation.[57] It is possible that enhanced GA biosynthesis in *elf3* arises from misregulation of genes for auxin biosynthesis and signaling that then leads to elevated expression of *GA20ox1* and *GA20ox2*. *YUCCA8* (*YUC8*) and *TAA1* are genes encoding enzymes for rate-limiting steps in auxin biosynthesis and each is subject to PIF4 regulation.[35,39,40] *YUC8* expression is significantly elevated in *elf3-2* compared to WT (**Fig. 3A**; unpaired t-test, $p < 0.05$); on the other hand, *TAA1* expression is unchanged in the *elf3* mutant background (**Fig. 3B**). The high *YUC8* expression in *elf3-2* is dependent on *PIF4* and *PIF5*, as *YUC8* transcript levels in the *elf3-2 pif4-101 pif5-1* triple mutant are below those in WT (**Fig. 3A**). Thus, PIF4 and PIF5 are responsible for elevated *YUC8* expression in the *elf3* mutant.

PIF4 and PIF5 also modify auxin sensitivity in hypocotyls to regulate growth.[57] To evaluate whether elevated auxin biosynthesis or signaling is apparent in the *elf3* mutant, the expression the *Aux/IAA* genes *IAA19* and *IAA29* was evaluated in WT and *elf3-2*. These *Aux/IAA* genes are regulated by PIF4 and PIF5.[58] Indeed, both *IAA19* and *IAA29* are highly upregulated in *elf3-2* (**Fig. 3C and D**; unpaired t-test, $p < 0.05$). Addition of the *pif4-101* and *pif5-1* alleles suppresses this phenotype, which indicates that *PIF4* and *PIF5* are needed for elevated *IAA19* and *IAA29* expression in the *elf3-2* background (**Fig. 3C and D**). These observations demonstrate that modified expression of auxin biosynthesis and signaling genes due to upregulation of PIF4 and PIF5 may underlie the GA-dependent growth phenotypes in *elf3*.

Discussion

Delineating connections between the circadian clock and hormone signaling is essential to understanding how plants time growth processes relative to predictable environmental events. GA is a fundamental regulator of many growth processes throughout all stages of the plant life cycle. An increasing number of examples show that the clock influences GA biosynthesis and signaling.[51-54] The findings here show that inactivating the EC alters expression of GA biosynthesis genes and that the defining growth phenotypes of Arabidopsis *elf3* mutants depend on GA biosynthesis. *elf3* mutants have high transcript levels of the GA biosynthesis genes *GA20ox1* and *GA20ox2*, which are normally tightly controlled to maintain GA homeostasis. Moreover, the timed presence of *GA20ox1* and *GA20ox2* transcripts in seedlings is strongly influenced by the transcription factors PIF4 and PIF5. In this role, PIF4 and PIF5 act redundantly. The similarities between the observations in Arabidopsis here and the demonstration that increased GA production in the barley *elf3* mutant drives elongated growth (see ref. 54), indicates that the EC has similar, if not identical, regulatory roles over GA biosynthesis and signaling in eudicots and monocots.

The findings here indicate that GA rhythms are conferred by EC-dependent nighttime repression of key GA biosynthesis genes and this is a fundamental means by which the clock regulates GA biosynthesis and GA-dependent growth. Defects in nighttime repression of barley GA metabolism genes occur when *ELF3* function is lost.[54] In SD photoperiods, the *GA20ox2* transcript level in WT barley increases just after dawn and continues to rise into the early part of the day, a time when EC activity is not present.[13,54] In contrast, *GA20ox2* expression in a barley *elf3* mutant was significantly higher throughout the night. The time of maximal difference between mutant and WT was in the pre-dawn to dawn time window (ZT21-0), at which time *GA2ox3* expression in *elf3* was about 4 times higher than in WT at pre-dawn (ZT21) but not near midday (ZT5). Corresponding to these expression changes, dawn levels of GA_{19}, GA_{20}, and GA_1, which are substrates and products of *GA20ox*, as well as the immediate degradation product GA_8, are all significantly higher in the barley *elf3* mutant.[54] Similar to *GA20ox2* in WT barley, Arabidopsis *GA20ox1* and *GA20ox2* expression rises after dawn and continues to increase for 4–8 hours when seedlings are grown under 12-hour day or LD conditions, while expression peaks at dawn in SD (**Fig. S1A and B**). Both *elf3-1* and *elf3-2* Arabidopsis mutants grown in short days have significantly higher *GA20ox1* and *GA20ox2* transcript abundance immediately before dawn compared to WT (**Fig. 2A and B**). In these conditions, the growth phenotypes of *elf3-1* are also particularly severe and GA biosynthesis is important for manifestation of these phenotypes, since the GA biosynthesis inhibitor PAC attenuates these (**Fig. 1**). Therefore, ELF3 regulates plant growth by affecting GA biosynthesis, presumably together with ELF4 and LUX in the EC.

In addition to being GA-dependent, enhanced hypocotyl and petiole growth in *elf3-1* requires the activity of PIF4 and PIF5. Together *pif4* and *pif5* mutant alleles restore normal hypocotyl length to *elf3* mutants (see ref. 13), as well as nearly WT expression for *GA20ox1* and *GA20ox2* (**Fig. 2**). Elevated *GA20ox1* and *GA20ox2* transcript levels in *elf3-1* likely arise from the enhanced PIF protein accumulation common to this mutant, especially in SD photoperiods.[25] Therefore, PIF4 and PIF5 positively affect *GA20ox1* and *GA20ox2* expression and this is revealed in the *elf3* mutant. Collectively, these results indicate that the clock regulates *PIF* gene expression to temporally control GA-dependent growth.

Considering how PIF protein activity is controlled in a GA-dependent manner, a feed-forward mechanism may enhance the growth phenotypes caused by EC inactivation. DELLA proteins repress GA signaling (for review see ref. 59), in part by direct binding of PIF proteins to block their activity.[28,60] This inhibition is attenuated in the presence of GA, by ubiquitin-26S proteasome mediated degradation of DELLA proteins.[61-64] In the

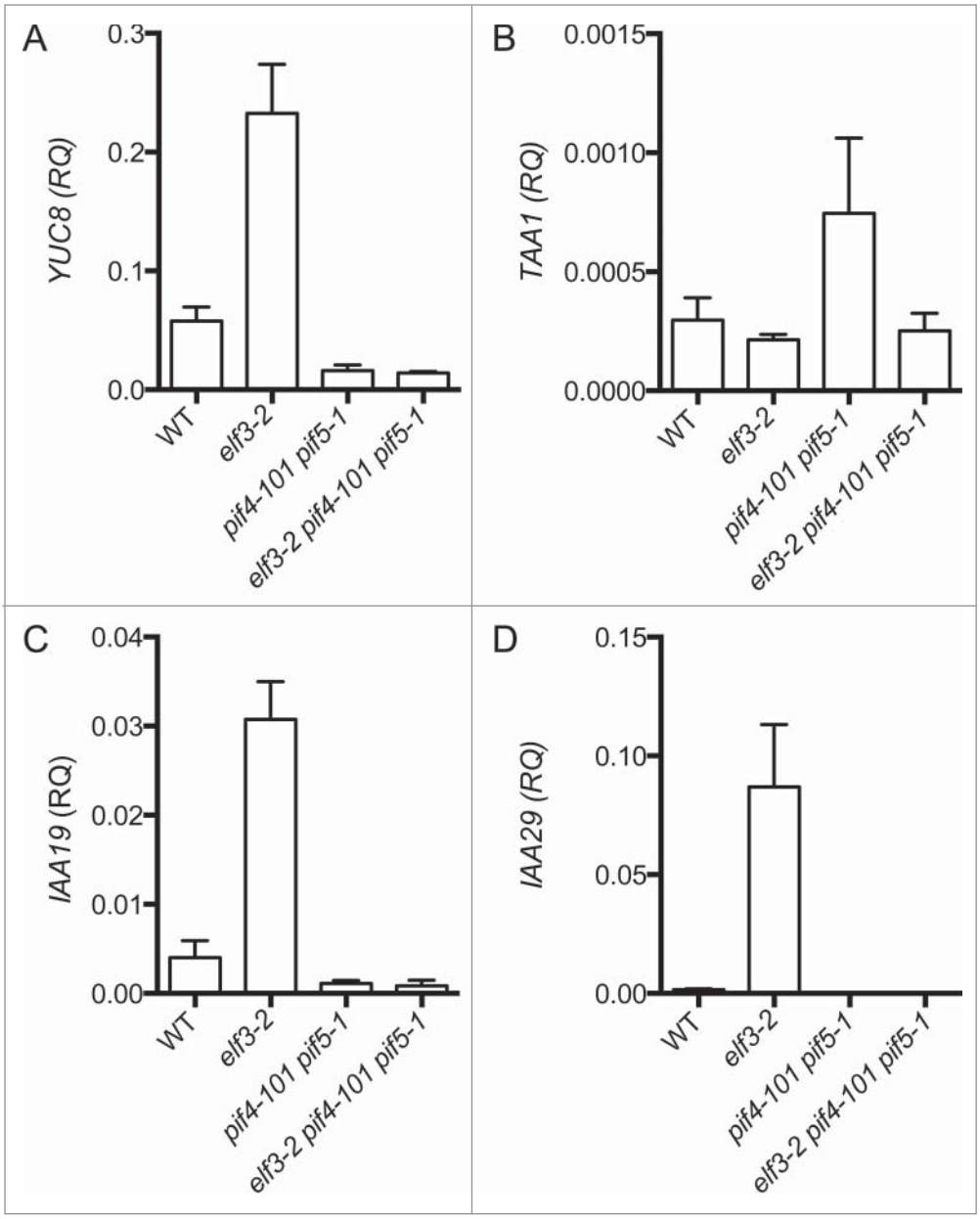

Figure 3. High expression of *YUC8*, *IAA19*, *IAA29* indicate potential elevated auxin biosynthesis and signaling in *elf3* mutants that requires *PIF4* and *PIF5*. Transcript abundance for (**A**) *TAA1*, (**B**) *YUC8*, (**C**) *IAA19* and (**D**) *IAA28* in the indicated genotypes at 15 minutes before ZT0 (lights-on) under SD conditions. Transcript levels were measured by qPCR analysis. Genotypes are depicted on x-axis and y-axis displays relative quantitation (RQ) of transcript normalized to *PDF2* according to Materials and Methods. Shown is the mean (±SEM) of 3 independent experimental replicates.

elf3 background, it is possible that the greater PIF protein abundance at an unusual time of day could overcome normal DELLA protein inhibition due a lack of sufficient DELLA to bind all PIF proteins; consequently, more PIF proteins could be available to activate GA biosynthesis. Furthermore, increased GA levels may maintain low levels of DELLA proteins, which can result in even more PIF activity.

Despite the fact that PIFs directly regulate many growth-related genes, PIF4 and PIF5 may not bind directly to the promoters of *GA20ox1* and *GA20ox2*. Maximum PIF4 and PIF5 protein accumulation occurs just before dawn, because the proteins are destabilized by light.[25,26] In contrast, *GA20ox* expression levels remain elevated into the daylight hours under 12-hour day and LD conditions, a time when PIF4 and PIF5 are at their lowest ebb (Supplementary **Fig. 1A and B**; see ref. 25). Furthermore, G-box or modified E-box sequences are not present in the promoter regions of *GA20ox1* and *GA20ox2* (B.C.T, unpublished observations).

Although a direct regulatory link for PIF4 and PIF5 is not established for *GA20ox1* and *GA20ox2*, loss of PIF4 and PIF5 activity impacts the expression of genes for auxin biosynthesis and signaling. *TAA1* and *YUC8* encode enzymes for the rate-limiting steps in auxin biosynthesis and each gene is subject to PIF4 and PIF5 control.[35,39,40] High *YUC8* expression in the *elf3* background is dependent on PIF4 and PIF5 (**Fig. 3A**). PIF4 and PIF5 also modify auxin sensitivity in hypocotyls to regulate growth.[57] Indeed, auxin-regulated genes are over-represented among genes differentially expressed between *pif4 pif5* double mutants and WT.[65,66] Auxin is an established regulator of GA biosynthesis for some plant processes, including hypocotyl elongation. Exogenous application of auxin increases transcript abundance for *GA20ox1*, *GA20ox2*, *GA3ox1*, and 3 of 7 *GA2ox* paralogs in Arabidopsis seedlings.[67,68] Furthermore, overexpression of *YUCCA1* produces elongated hypocotyls and this phenotype is suppressed by PAC treatment.[67] Thus, auxin-dependent hypocotyl growth requires GA biosynthesis and PAC inhibits the effects of exogenous auxin. Circadian-regulated genes are over-represented in auxin responsive genes, with peak phases at pre-dawn to dawn (ZT22-2), which are times when hypocotyl growth is most active.[57,69] In *elf3-2*, the Aux/IAA genes *IAA19* and *IAA29* are highly expressed only when PIF4 and PIF5 are active (**Fig. 3C and D**). Therefore, the EC potentially acts

through PIF4 and PIF5 to control auxin biosynthesis and this module ultimately regulates GA biosynthesis.

The work presented here establishes an EC-dependent role for GA biosynthesis in control of elongation growth in Arabidopsis. Photoperiod-insensitive early flowering, however, is another defining aspect of *elf3* mutants in Arabidopsis, barley, and pea.[45,54,65] An established cause of this early flowering phenotype is constitutively high *CONSTANS* (CO) expression that promotes high expression of *FLOWERING LOCUS T* (*FT*)[70]. Since recent findings show that PIF4, and possibly PIF5, directly bind to the FT promoter to accelerate flowering (see refs. 27, 71), the high PIF levels throughout the night in *elf3* mutant backgrounds may also result in elevated *FT* expression under some environmental conditions, especially those that include elevated temperature.[71] Alternatively, PIF-dependent accumulation of GA may also induce flowering through a GA-dependent flowering pathway.[72] The early flowering phenotype in barley *elf3* is GA-dependent and some flowering time genes respond to GA.[54] While in the past, flowering in *elf3* has been largely attributed to mis-regulation of the photoperiod pathway and CO, this classic early flowering mutant can now be re-examined for its reproductive defects in the context of PIF transcription factors and GA.

This work demonstrates that many factors work together in a temporally regulated module to control plant growth. These circadian clock-hormone modules are an importation aspect of plant biology, as plants must time growth and developmental processes relative to daily environmental cycles. While the mechanistic role that ELF3 plays in the circadian clock is increasingly better understood in recent years, the connections between the clock and physiological outputs remain at the forefront of many investigations in chronobiology.

Methods

Plant growth

All plants were in the Columbia-0 (Col-0) background. The *elf3-2* mutant is described by Nusinow et al.[42] *pif4-2* and *pif5-3* were a gift from Dr. Peter Quail (Plant Gene Expression Center, Albany, CA, USA; see refs. 73, 74), and *elf3-2*, *pif4-101 pif5-1,* and *elf3-2 pif4-101 pif5-1* seeds were a gift from Dr. Dmitri Nusinow (Donald Danforth Plant Science Center, St. Louis, MO, USA). Seeds for all experiments were surface-sterilized in a 50% bleach solution and stratified for 2–5 d in the dark at 4°C before use. All seedlings were grown in Percival growth chambers (Percival Scientific, www.percival-scientific.com), set to constant 22°C and SD conditions (8 h light:16 h darkness) with cool white light at 100 μmol/m^2/s.

PAC treatments

Seeds were sown at ZT0 on 100 mm × 15 mm square plates (BD Biosciences, www.bdbiosciences.com) containing 50 ml of MS medium at pH 5.8 with 0.8% type I micropropagation agar (Caisson Laboratories, www.caissonlabs.com). For hypocotyl measurements, seedlings were grown for two full days on MS plates and then were transferred at ZT0 on day 3 under sterile conditions to either MS plates or MS plates containing paclobutrazol (Chem Service, www.chemservice.com) at concentrations ranging from 0.1 to 10 μM. Seedlings were allowed to grow for 5 additional days, then images of seedlings were made by scanning the plates with a flatbed scanner and hypocotyl length calculated from the images using ImageJ software (http://imagej.nih.gov/ij/). For petiole measurements, seeds were sown into Phytatray boxes (Sigma Aldrich, www.sigmaaldrich.com) containing 250 mL MS media as above. After one week, seedlings were transferred to boxes containing paclobutrazol concentrations ranging from 0 to 10 μM. After an additional 3 weeks at these conditions, images were taken of the plants and measurements made from the images using ImageJ software.

Quantitative real-time PCR

Seeds for qPCR experiments were sown onto sterile filter paper (Whatman, www.whatman.com) and grown on MS plates as above. Fifteen minutes before ZT0, while seedlings were in the dark, entire seedlings were harvested under a green LED light, placed into 1.5 ml microcentrifuge tubes, and immediately frozen in liquid N_2. Frozen samples were pulverized in 1.5 mL tubes with micropestles. RNA was isolated with the Qiagen RNeasy Kit and contaminating genomic DNA digested with the Qiagen RNase-free DNase Set according to the manufacturer's protocol (Qiagen, www.qiagen.com/us/). First-strand cDNA was prepared from 1 μg of total RNA with the Maxima Universal First Strand cDNA Synthesis kit (Thermo Scientific, www.thermofisher.com/) and diluted 1:5 in RNase-free water prior to use. Transcript levels were determined with qPCR using Bio-Rad SsoAdvanced Universal Supermix and a CFX96 Real-Time PCR Detection System according to manufacturer's protocols (Bio-Rad, www.bio-rad.com). Transcript levels of target genes were calculated using the equation $RQ = 2^{[Ct\ (Control)\ -\ Ct\ (Experimental)]}$, where C_t is the mean threshold cycle for each sample. The transcript from *PDF2* served as the normalization control (AT1G13320).[75] Primer sequences are in **Table S1**.

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Supplementaral Materials

Supplemental data for this article can be accessed on the publisher's website.

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