Genomic Analysis of Circadian Clock-, Light-, and Growth-Correlated Genes Reveals PHYTOCHROME-INTERACTING FACTOR5 as a Modulator of Auxin Signaling in Arabidopsis[1][C][W][OA]

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Plants exhibit daily rhythms in their growth, providing an ideal system for the study of interactions between environmental stimuli such as light and internal regulators such as the circadian clock. We previously found that two basic loop-helix-loop transcription factors, PHYTOCHROME-INTERACTING FACTOR4 (PIF4) and PIF5, integrate light and circadian clock signaling to generate rhythmic plant growth in Arabidopsis (*Arabidopsis thaliana*). Here, we use expression profiling and real-time growth assays to identify growth regulatory networks downstream of PIF4 and PIF5. Genome-wide analysis of light-, clock-, or growth-correlated genes showed significant overlap between the transcriptomes of clock-, light-, and growth-related pathways. Overrepresentation analysis of growth-correlated genes predicted that the auxin and gibberellic acid (GA) hormone pathways both contribute to diurnal growth control. Indeed, lesions of GA biosynthesis genes retarded rhythmic growth. Surprisingly, GA-responsive genes are not enriched among genes regulated by PIF4 and PIF5, whereas auxin pathway and response genes are. Consistent with this finding, the auxin response is more severely affected than the GA response in *pif4 pif5* double mutants and in *PIF5*-overexpressing lines. We conclude that at least two downstream modules participate in diurnal rhythmic hypocotyl growth: PIF4 and/or PIF5 modulation of auxin-related pathways and PIF-independent regulation of the GA pathway.

Proper control of plant growth is essential for determining shape and size, fundamental properties for agriculture, biomass production, and ecological adaptation. Plant growth is controlled by both external and internal cues (such as light and the circadian clock, respectively), but the growth-control system is so complex that our understanding of how these cues are integrated for growth control is limited (Nozue and Maloof, 2006; Alabadí and Blázquez, 2009).

Light is an important regulator of plant growth; early in development, it inhibits the elongation of the young plant stem, or hypocotyl. This response is mediated by phytochromes and cryptochromes, photoreceptors that sense red and far-red light or blue light, respectively (Jiao et al., 2007). Stem elongation is also controlled by the plant circadian system, a complex transcriptional network that affects many aspects of plant physiology and growth (Lecharny and Wagner, 1984). When seedlings are maintained in constant environmental conditions, growth is clock regulated such that rapid hypocotyl elongation occurs at the end of the subjective day but little or no growth occurs during the subjective morning (Dowson-Day and Millar, 1999).

The light and clock pathways interact to control stem elongation in normal light/dark cycles. We recently showed that when plants are grown in short days, maximal hypocotyl elongation occurs in the morning with little or no growth occurring in the evening (Nozue et al., 2007). Disruption of either clock function or light signaling alters this rhythmic pattern. Investigating the mechanism underlying normal diurnal growth, we found that the clock and light signaling pathways converge to control two transcription factors (TFs) via transcriptional and posttranscriptional regulation. Growth initiation late in the night is due to clock-regulated induction of *PHYTOCHROME-INTERACTING FACTOR4* (*PIF4*) and *PIF5* transcripts. Growth ceases in the morning because light causes degradation of the PIF4 and PIF5 proteins. Growth remains quiescent in the first part of the night because at this time the clock inhibits *PIF4* and *PIF5* transcription (Nozue et al., 2007). The coordinated regulation of PIF4 and PIF5 is also responsible for the photoperiodic

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control of hypocotyl elongation (Niwa et al., 2009). However, the transcriptional targets downstream of PIF4 and PIF5 that cause these daily growth rhythms have yet to be identified.

Possible targets of PIF4 and PIF5 action include hormone signaling pathways. Plant hormones are small molecules that are important for growth, development, and regulation of physiology. Among them, GA, auxin, and brassinosteroids (BRs) play major roles in growth control. Binding of GA to its receptor proteins triggers ubiquitin-mediated degradation of the DELLA signaling proteins, triggering changes in the expression of GA-regulated genes (Schwechheimer, 2008). The DELLA proteins have been shown to physically interact with PIF4 and modulate its activity (de Lucas et al., 2008). Similarly, altered PIF4 expression has been reported to change GA sensitivity (de Lucas et al., 2008), making the GA pathway a candidate mediator of PIF4-controlled daily growth rhythms.

Auxin and BRs are known to have important roles in many aspects of plant growth and development (Vanneste and Friml, 2009; Kim and Wang, 2010). Similar to GA signaling, binding of auxin to the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX receptors triggers ubiquitin-mediated degradation of AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (AUX/IAA) proteins (Mockaitis and Estelle, 2008). AUX/IAA proteins normally interact with and inactivate TFs known as auxin response factors. Auxin promotion of AUX/IAA protein degradation causes the release of active auxin response factors that in turn bind to auxin-responsive promoter elements to activate or repress downstream gene expression. A different signaling mechanism is utilized in the BR pathway. BR is perceived by the BR INSENSITIVE1 receptor kinase, leading to phosphorylation and dephosphorylation of downstream proteins and finally changes in transcriptional activity (Kim and Wang, 2010). Other important control mechanisms in the GA, auxin, and BR pathways include their regulated biosynthesis and inactivation (Yamaguchi, 2008; Kim and Wang, 2010; Normanly, 2010). For auxin, changes in hormone transport are key regulatory steps as well (Grunewald and Friml, 2010).

Direct connections between light, circadian, and hormone signaling pathways have already been made. Free auxin levels are clock regulated (Jouve et al., 1998; Rawat et al., 2009), and in addition, plant sensitivity to auxin varies in a circadian manner (Covington and Harmer, 2007). As expected given these findings, genes encoding auxin signaling components and auxin-responsive genes show a higher incidence of circadian regulation than expected by chance (Covington and Harmer, 2007). The GA and BR pathways are also likely regulated by the circadian clock, since genes regulated by these hormones are also significantly enriched for circadian regulation (Dodd et al., 2007; Covington et al., 2008; Michael et al., 2008).

There are also multiple links between light and hormone signaling. Light causes changes in the expression of auxin signaling genes (Hoecker et al., 2004; Tian et al., 2004; Tao et al., 2008) and alters auxin-dependent root development (Salisbury et al., 2007) and apical hook formation (Li et al., 2004). In addition, the effect of GA on hypocotyl growth is different in dark- and light-grown seedlings (Cowling and Harberd, 1999). In plants grown in short days, genes regulated by auxin, BR, and GA tend to show highest expression at dawn, the time of maximal hypocotyl growth (Michael et al., 2008). This suggests that coordinated regulation of these hormone pathways by light and the circadian clock might underlie the observed daily growth rhythms.

Despite these findings, an integrated genome-wide understanding with light, the clock, and hormone pathways, as well as pathways downstream of *PIF4* and *PIF5* in plant growth regulation, is still lacking. To better understand how PIF4 and PIF5 integrate information from the light and clock pathways to regulate plant growth, we have performed whole-genome time-course expression and kinetic analysis of growth. Using wild-type, clock mutant, and growth-defective *pif4 pif5* mutant plants, we have identified genes whose expression patterns are correlated with Arabidopsis (*Arabidopsis thaliana*) hypocotyl growth. Among growth-correlated genes, we found significant overlap between light, clock, and hormone transcriptomes. In particular, the growth-correlated gene list is statistically significantly enriched for genes in the auxin, GA, and BR plant hormone pathways. Mutant analysis suggests that although GA is required for growth promotion by PIF4 and PIF5, these TFs do not affect plant responsiveness to GA. In contrast, our data indicate that auxin responsiveness is altered in plants with altered PIF4 and PIF5 function. Based on these bioinformatic and physiological analyses, we conclude that one way in which PIF4 and PIF5 integrate information from light and the circadian clock is by modulating the auxin pathway.

RESULTS

We wish to understand how PIF4 and PIF5 integrate information from the light and clock pathways to regulate plant growth. To this end, we have used bioinformatic analyses to identify growth-related genes regulated by interactions between light signaling and the circadian clock. We first present an analysis of clock-light interactions. This is followed by microarray analysis of growth-correlated genes, where we first validate our methods by looking for known growth-related genes and then identify new candidate growth-related genes. Finally, we investigate which of these genes may be regulated by PIF4 and PIF5 and test our predictions in plant growth assays.

Genome-Wide Analysis of Light and Clock Interactions

Because of the extensive cross talk between the light and clock pathways, we used existing data to examine

this interaction in a genomics context. Defining light-responsive genes as those up- or down-regulated by any type of light treatment (i.e. pulse or continuous, short or long exposure of monochromatic [red/far-red/blue] light), we made a list of light-responsive genes using data from publicly available microarray experiments (Supplemental Table S1; see "Materials and Methods"). We next determined which of these genes also have clock-regulated expression in constant light (Covington et al., 2008; Supplemental Table S2) and then calculated the distribution of peak expression in circadian time. Although clock-regulated genes showed a relatively even distribution of times of peak expression, genes up-regulated by light were most likely to have peak expression during the subjective morning, whereas genes down-regulated by light tended to have peak expression in the subjective evening (Fig. 1). This finding is consistent with a recent smaller scale analysis (Usadel et al., 2008) and suggests that gating of light-responsive genes may be extensive, with most light-induced genes being gated for morning induction and light-repressed genes for evening expression, a phenomenon observed for many environmental responses (Hotta et al., 2007).

We next wished to identify signaling pathways that are regulated both by light and the clock (Supplemental Table S3) using overrepresentation analysis (ORA) to look for categories of genes present more than expected by random chance. Such genes could be downstream outputs or key regulators of light-clock interactions. ORA of light-induced and clock-regulated genes showed a significant enrichment for genes involved in photosynthesis and secondary metabolism but not for those involved in hormone signaling (Supplemental Table S3). In contrast, among all genes that are both light repressed and clock regulated, we found that auxin-responsive genes were highly enriched (Supplemental Table S3).

We next asked if this enrichment of auxin-responsive genes is specific to those genes regulated by both light and the clock. ORA showed that auxin up-regulated genes were overrepresented both in light-repressed and clock-regulated genes (Supplemental Table S3C), consistent with previous studies (Tepperman et al., 2006; Covington et al., 2008). To test whether there was additional enrichment among the intersection of genes both regulated by the clock and repressed by light (clock∩light_rep.), we tested whether any auxin categories were overrepresented in the clock∩light_rep. genes relative to the individual clock- and light-repressed sets. There is no additional enrichment of auxin up-regulated genes in the clock∩light_rep. genes when compared with all light-responsive genes; however, there is an additional enrichment in the clock∩light_rep. genes when compared with all clock-regulated genes (underlined in Supplemental Table S3C). This likely reflects the higher proportion of auxin up-regulated genes among light-repressed genes. In contrast to the results for auxin up-regulated genes, genes implicated in auxin signaling (BINCODE 3 in Supplemental Table S4), while overrepresented in clock-regulated genes as reported previously (Covington and Harmer, 2007) and among light-responsive genes, were not enriched in the intersection (only two genes). Thus, while both the clock and light networks regulated the auxin pathway (shown by the overrepresenation of auxin pathway and auxin-regulated genes), the auxin signaling pathway is regulated differently by the light and clock networks; furthermore, the enrichment of auxin-responsive genes among genes regulated by both light and the clock is simply due to their enrichment in the individual sets.

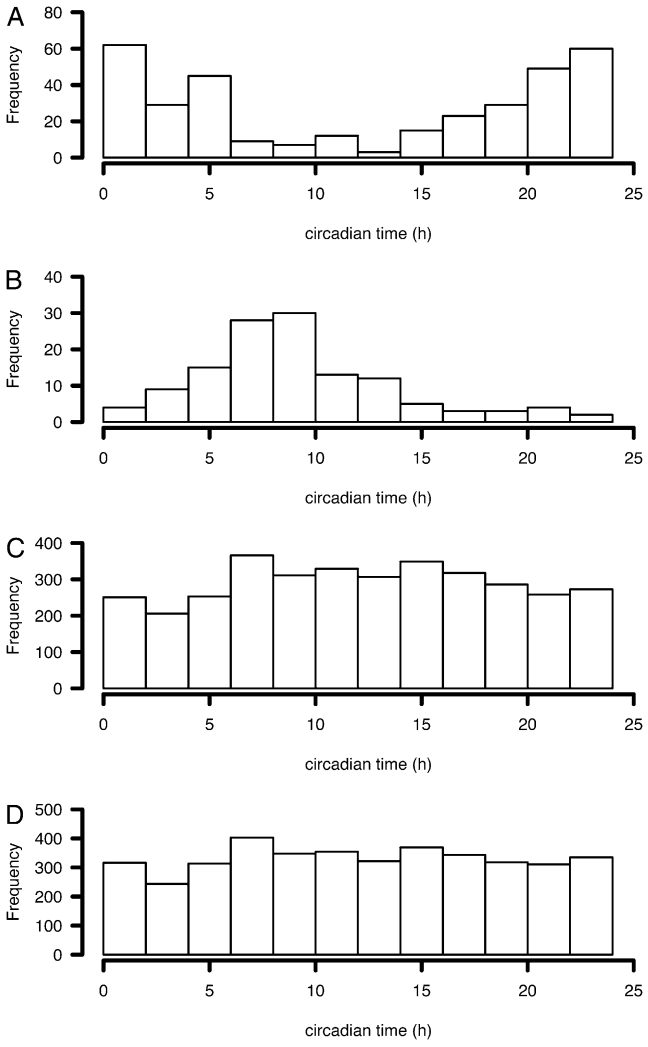

Figure 1. Phase distribution of clock- and light-regulated genes. The times of peak circadian expression are depicted. A, Genes up-regulated by light. B, Genes down-regulated by light. C, Non-light-responsive genes. D, All circadian clock-regulated genes (Covington et al., 2008). The number of genes in each column is labeled on the *y* axis. For circadian time (h), 0 corresponds to subjective dawn and 12 to subjective dusk.

Design of Microarray Experiments

We recently demonstrated that a powerful method for identifying genes controlling hypocotyl growth was by the identification of genes whose expression patterns correlate with growth patterns by genome-wide expression analysis (Nozue et al., 2007). We compared gene expression in plants with arrhythmic circadian clocks (due to overexpression of the clock gene *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*); Wang and Tobin, 1998) and wild-type plants at different times of day. We now extend this study with a more detailed gene expression analysis that includes more time points and an additional genotype, the *pif4 pif5* mutant. We chose this double mutant because single *pif4* or *pif5* mutants have a relatively mild phenotype, likely due to redundancy (Nozue et al., 2007). We entrained plants in short days, and then transferred them to SD/3 conditions (artificially short light/dark cycles) and collected samples over multiple days (Supplemental Fig. S1). We chose SD/3 instead of short-day conditions because growth, light, and circadian regulation are confounded in short days. Specifically, in short-day conditions, ecotype Columbia (Col) only grows at the end of the night, confounding growth genes with those normally expressed at this time of night because of the phase of the circadian clock, the absence of light, or the duration of the dark period. Similarly, if we are interested in genes expressed in plants that are in the light but not growing, short-day conditions limit our collection times to 4 to 8 h after dawn. The *CCA1-OX* genotype helps alleviate confounding effects of the circadian clock, and the SD/3 conditions help alleviate the confounding effects of light and dark period duration. This experimental paradigm allowed us to correlate gene expression with growth both within a genotype (i.e. Col at 280 min versus 1,240 min) and across genotypes (i.e. Col versus *CCA1-OX* at 1,240 min), as described previously (Nozue et al., 2007). The additional time points in this study provide more statistical power, allowing us to identify a more comprehensive network of growth-correlated genes. We used Rank Product analysis (Breitling et al., 2004; see "Materials and Methods") to identify the genes differentially expressed in growing phases (up in growing phase [$_{up}$G]) or stationary phases (up in stationary phase [$_{up}$S]). Genes were considered differentially expressed if they had a false discovery rate (FDR) less than 0.01 and a fold change less than 0.9 or greater than 1.1. We will refer to the roughly 2,000 genes (Supplemental Table S5) in these categories as growth-correlated genes.

Known Growth Genes in the Growth-Correlated Gene List

To validate the growth-correlated gene list, we asked if the list contained known growth-related genes. Indeed, the gene lists contain numerous known growth genes (Supplemental Fig. S2; Supplemental Table S4; Anastasiou and Lenhard, 2007; Busov et al., 2008; Krizek, 2009), including genes involved in hormone-, light signaling-, and circadian clock-related pathways as well as TFs implicated in growth control. These genes are discussed in detail in Supplemental Results S1.

We also used ORA to determine whether particular gene categories or genetic pathways are enriched among the growth-correlated genes (Supplemental Table S6). We found that genes implicated in several hormone pathways are overrepresented in the $_{up}$G category, whereas none of them was overrepresented in $_{up}$S (Supplemental Table S6A). In particular, genes related to metabolism, transport, or signaling ("hormone pathway" genes hereafter) of auxin, GA, BR, and ethylene are enriched in the $_{up}$G category. These four hormones are all important regulators of plant growth. By contrasting growing and nongrowing plants in SD/3 conditions and using both wild-type and arrhythmic *CCA1-OX* plants, we are able to untangle the effects of the circadian clock and light input and more directly identify growth-correlated genes than previous studies. Our analysis confirms and extends the analysis by Michael et al. (2008), in which they identified enriched expression of auxin-, GA-, BR-, and abscisic acid-regulated genes at times of rapid growth in wild-type plants grown in short-day conditions. The specific genes in these pathways with peak expression correlated with growth (i.e. candidate mediators of growth processes) are discussed in detail in Supplemental Results S1. In addition, we found that genes regulated by these hormone pathways are significantly enriched in the $_{up}$G gene list (Supplemental Table S7; Supplemental Results S1), suggesting that the activities of these hormone pathways are involved in the coordinated regulation of growth by the light and clock networks. The identification of many known growth-related genes in our lists suggests that novel growth-control genes may also be present.

ORA to Find New Growth-Control Candidate Genes

Having validated our $_{up}$G and $_{up}$S gene lists as containing many growth-correlated genes, we next used ORA to define new candidate growth-regulating genes. Specifically we reasoned that gene categories or genetic pathways that are enriched in the growth-correlated gene sets (Tables I and II; Supplemental Table S6) would provide good candidates for new growth regulators, especially when the MapMan category contained other known growth regulators. Whereas above we discussed known growth regulators in the $_{up}$G and $_{up}$S lists, below we detail new candidate genes and pathways.

Five TF subgroups (subgroups of BINCODE 27.3 in Tables I and II) were enriched in $_{up}$G, while one subgroup was enriched in $_{up}$S. Genes in some of these subgroups have been shown to control hypocotyl growth (see above), so other TF genes in these subcategories are strong candidates for new growth-controlling

Table I. *ORA of growth phase-correlated genes (genes with higher expression during growth phases; $_{up}$G) by PageMan analysis*

MapMan bins with $P < 0.0005$ are shown. Specific gene families (hormone pathways, cell wall biosynthesis genes, and ABC transporters) are provided in Supplemental Table S1. Ratio represents number of genes in a bin found divided by expected number of genes in the bin.

MapMan Bin	Bin Name	*P*	Ratio
28.1	DNA.synthesis/chromatin structure	4.64E-26	0.05
17	Hormone metabolism	1.38E-25	5.02
28	DNA	1.70E-24	0.1
17.2	Hormone metabolism.auxin	4.41E-15	6.48
17.2.3	Hormone metabolism.auxin.induced-regulated-responsive-activated	1.51E-14	7.1
27.3	RNA.regulation of transcription	3.32E-14	2.09
35	Not assigned	3.05E-13	0.68
29.2	Protein.synthesis	1.20E-10	0.1
10	Cell wall	5.81E-10	3.16
27	RNA	1.15E-09	1.76
17.5	Hormone metabolism.ethylene	1.71E-09	6.77
27.3.40	RNA.regulation of transcription.Aux/IAA family	8.41E-09	13.82
10.7	Cell wall.modification	1.36E-08	7.59
17.5.2	Hormone metabolism.ethylene.signal transduction	1.07E-07	10.66
35.2	Not assigned.unknown	1.32E-07	0.7
29	Protein	1.46E-06	0.6
30.11	Signaling.light	1.98E-06	5.47
27.3.7	RNA.regulation of transcription.C2C2(Zn) CO-like, Constans-like zinc finger family	7.35E-06	9.36
27.3.6	RNA.regulation of transcription.bHLH,Basic Helix-Loop-Helix family	9.40E-06	4.09
3.2.3	Minor CHO metabolism.trehalose.potential TPS/TPP	1.11E-05	23.69
26	Misc.	1.92E-05	1.75
27.3.66	RNA.regulation of transcription.Pseudo ARR transcription factor family	3.85E-05	18.42
17.6	Hormone metabolism.gibberellin	1.09E-04	6.31
34.13	Transport.peptides and oligopeptides	1.19E-04	5.35
35.1	Not assigned.no ontology	1.22E-04	0.66
3.2	Minor CHO metabolism.trehalose	1.51E-04	9.42
17.5.1	Hormone metabolism.ethylene.synthesis-degradation	3.20E-04	6.38
17.6.3	Hormone metabolism.gibberellin.induced-regulated-responsive-activated	3.71E-04	11.05
26.12	Misc. peroxidases	4.10E-04	4.48
34.2	Transporter.sugars	4.10E-04	4.48
27.3.20	RNA.regulation of transcription.G2-like transcription factor family, GARP	4.23E-04	6.07
29.2.1	Protein.synthesis.mito/plastid ribosomal protein	4.56E-04	0.09
20.2	Stress.abiotic	4.66E-04	2.24

genes (Tables I and II; Supplemental Table S5). Since transcriptional regulatory proteins generally have short half-lives (Collins and Tansey, 2006), it is possible that the transcripts and proteins encoded by these genes show similar rhythms in abundance and may be responsible for the observed growth rhythms.

Regulated protein degradation is essential in light (Henriques et al., 2009) and hormone signaling (Dreher and Callis, 2007) and for circadian clock function (Más, 2008). ORA revealed significant underrepresentation of protein degradation-related genes (Skp, Cullin, F-box complex) in $_{up}$S (BINCODE 29.5.11.4.3 in Table II). Protein degradation is a critical step for posttranscriptional regulation (Stone and Callis, 2007); however, our results suggest that transcriptional control of this machinery is not important for growth regulation. Furthermore, the significant underrepresentation hints that relatively constant transcription of the protein degradation machinery could be important.

Other candidate genes, such as those involved in xyloglucan:xyloglucosyl transferase, lignin biosynthesis, and white-brown complex subfamily ATP-binding cassette (ABC) transporters, which may be targets of growth regulatory factors, are described in Supplemental Results S1.

Light and Growth Interactions

Since light is an important regulator of plant growth, we asked about the relation between light-regulated genes and those we found to be growth correlated. Light-responsive genes were significantly enriched in the growth-correlated gene sets (both $_{up}$G and $_{up}$S), as

Table II. *ORA of stationary phase-correlated genes (genes with higher expression during stationary phases; $_{up}$S) by PageMan analysis*
See Table I legend for details. SCF, Skp, Cullin, F-box.

MapMan Bin	Bin Name	*P*	Ratio
28.1	DNA.synthesis/chromatin structure	9.24E-40	0.03
28	DNA	5.48E-37	0.08
16	Secondary metabolism	2.17E-22	4.46
35.2	Not assigned.unknown	1.79E-17	0.6
26	Misc.	1.95E-15	2.21
16.2.1	Secondary metabolism.phenylpropanoids.lignin biosynthesis	3.36E-14	12.25
34	Transport	6.57E-14	2.43
16.2	Secondary metabolism.phenylpropanoids	1.82E-13	7.25
35	Not assigned	1.38E-11	0.76
29.2	Protein.synthesis	1.46E-10	0.22
20.2	Stress.abiotic	2.00E-10	2.99
29	Protein	1.42E-09	0.58
20.2.1	Stress.abiotic.heat	1.51E-09	4.05
17	Hormone metabolism	8.83E-09	2.55
29.5.11.4.3	Protein.degradation.ubiquitin.E3.SCF	6.92E-08	0.15
29.5.11	Protein.degradation.ubiquitin	7.82E-08	0.36
29.5.11.4.3.2	Protein.degradation.ubiquitin.E3.SCF.FBOX	2.00E-07	0.16
33.2	Development.late embryogenesis abundant	2.17E-07	9.45
27.3.26	RNA.regulation of transcription.MYB-related transcription factor family	4.33E-07	6.78
16.8	Secondary metabolism.flavonoids	5.77E-07	4.72
16.4.1	Secondary metabolism.N misc.alkaloid-like	7.18E-07	9.86
13.2	Amino acid metabolism.degradation	1.26E-06	5.12
35.1.1	Not assigned.no ontology.ABC1 family protein	2.64E-06	13.08
16.4	Secondary metabolism.N misc	3.89E-06	8.1
20	Stress	4.72E-06	1.77
16.2.1.7	Secondary metabolism.phenylpropanoids.lignin biosynthesis.CCR1	7.50E-06	22.67
33	Development	9.81E-06	2.02
34.16	Transport.ABC transporters and multidrug resistance systems	1.32E-05	3.51
35.1.22	No ontology.late embryogenesis abundant domain-containing protein	2.10E-05	12.88
26.1	Misc. cytochrome P450	2.21E-05	2.66
29.5.11.4	Protein.degradation.ubiquitin.E3	2.76E-05	0.44
29.5.7	Protein.degradation.metalloprotease	2.95E-05	6.3
2	Major CHO metabolism	4.11E-05	3.76
29.5	Protein.degradation	4.25E-05	0.57
14.2	S-assimilation.APR	4.38E-05	28.34
13.2.5.2	Amino acid metabolism.degradation.serine-glycine-cysteine group.glycine	4.96E-05	16.19
11	Lipid metabolism	6.46E-05	2.21
16.1.3	Secondary metabolism.isoprenoids.tocopherol biosynthesis	9.64E-05	14.17
17.3.1.2.99	Hormone metabolism.brassinosteroid.synthesis-degradation.sterols.other	9.64E-05	14.17
1	Photosynthesis	1.29E-04	2.71
13.2.5	Amino acid metabolism.degradation.serine-glycine-cysteine group	1.69E-04	12.59
16.2.1.1	Secondary metabolism.phenylpropanoids.lignin biosynthesis.PAL	1.71E-04	21.25
18.5.2	Cofactor and vitamin metabolism.folate & vitamin K.vitamin K	1.71E-04	21.25
27.3	RNA.regulation of transcription	2.32E-04	1.4
16.8.3	Secondary metabolism.flavonoids.dihydroflavonols	2.39E-04	6.54
21.2	Redox.ascorbate and glutathione	2.41E-04	4.25
30.11	Signaling.light	3.62E-04	3.43
34.3	Transport.amino acids	3.96E-04	3.99

shown by Fisher's exact test (Supplemental Table S2). These genes may control growth in response to light transitions. ORA of the intersection between light-repressed and $_{up}$G genes (Supplemental Table S8) showed enrichment of auxin-related genes, cell wall genes, three TF subgroups (C3H zinc finger, homeobox, and bHLH), and GA-related genes. ORA of intersections between light-induced and $_{up}$S genes (Supplemental Table S8) showed enrichment of flavonoid biosynthesis genes, abiotic stress-related genes, three TF subgroups (CONSTANS [CO]-like, MYB-related, and APETALA2/EREBP), cell wall genes, light signaling genes, and photosynthesis-related genes.

The shade-avoidance syndrome is another type of light response. Plants can sense their neighbors by a shift in the ratio of red to far-red light. Plants undergoing shade avoidance grow faster, have more apical dominance, and flower earlier (Franklin, 2008). In other words, plants can perceive the quality of light conditions (shade) as well as binomial light conditions

(light on or off). We first asked how much overlap there was between genes defined as light responsive in seedlings exposed to monochromatic light ("light responsive" as discussed previously) and genes regulated by shade. We found that light-responsive genes were significantly enriched in shade-responsive genes but that the two sets only partially overlapped (Supplemental Table S9C). $_{up}$G genes were highly overrepresented in shade-induced genes and $_{up}$S genes were highly overrepresented in shade-repressed genes (Supplemental Table S9D), which is consistent with the fact that shade induces growth. This intersection of $_{up}$G or $_{up}$S genes with shade-responsive genes provides strong candidates for regulators of shade-induced growth.

Interactions between the Circadian Clock and Growth

Because of the importance of the circadian clock in growth rhythms, we examined the relationship between the clock and growth-correlated genes. Clock-regulated genes were enriched in both the $_{up}$G and $_{up}$S sets (Supplemental Table S2). Since the clock is important for rhythmic growth under both diurnal and constant light conditions, we hypothesized that the temporal expression patterns of $_{up}$G and $_{up}$S genes should correlate with growth not only under diurnal and SD/3 conditions but also under constant light. If this is true, then in constant light, $_{up}$G and $_{up}$S gene expression levels should peak at subjective dusk and dawn, respectively (Dowson-Day and Millar, 1999). To test this hypothesis, we examined the distribution of peak expression times among circadian-regulated genes that are in our growth-correlated gene lists and found that, indeed, $_{up}$G genes are enriched for dusk-phased expression and $_{up}$S genes are enriched for dawn-phased expression (Fig. 2A).

Since our samples under SD/3 conditions contain those from subjective dusk (280, 1,720, and 3,160 min after the dawn of day 3 after seeds were transferred to the incubator; Supplemental Fig. S1), the results shown in Figure 2A could simply reflect the time of sampling. In other words, perhaps dusk genes are enriched in the $_{up}$G set because $_{up}$G includes samples taken in the (subjective) evening. To test this possibility, we reanalyzed our data, excluding samples from subjective dusk to eliminate possible artifacts. Even in this new data set (which has 98% or 94% overlap with $_{up}$G or $_{up}$S, respectively), evening genes and morning genes were enriched among these smaller $_{up}$G or $_{up}$S gene sets, respectively (Fig. 2B).

Another possible explanation for the observed circadian time distribution of peak gene expression is that it is caused by the enrichment of light-responsive genes in $_{up}$G and $_{up}$S (Fig. 1). To test this possibility, we compared the distribution of peak expression times between light-responsive and nonresponsive genes in the reduced $_{up}$G or $_{up}$S sets used for Figure 2B. There were no apparent effects of light responsiveness on the distribution (Fig. 2, C and D), suggesting that our original hypothesis is correct: the clock regulates the transcription of growth-correlated genes independent of either sampling time or light signaling. This also confirms that our identification of growth-correlated genes is not dependent upon light regulation.

To identify the types of growth genes controlled by the clock, we performed ORA on growth-correlated and clock-regulated genes (Supplemental Table S10). Auxin-related genes, some TF subgroups (bHLH, PSEUDO-RESPONSE REGULATOR, and CO-like), and trehalose metabolism were enriched in the intersection between $_{up}$G and clock-regulated genes. Lignin biosynthesis genes, flavonoid biosynthesis genes, TF subgroups (CO-like and MYB-related), carbohydrate (CHO) metabolism genes (starch degradation), and amino acid metabolism were enriched in the intersec-

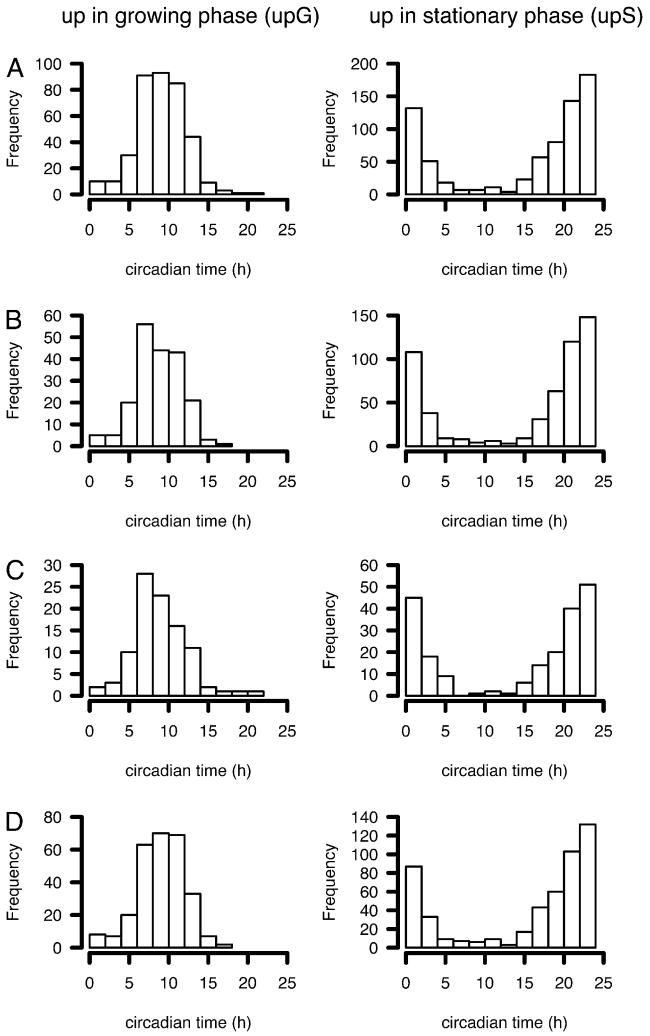

Figure 2. Interaction between the circadian clock and growth. The number of genes (*y* axis) with peak expression at a particular circadian time (*x* axis) is shown for all $_{up}$G or $_{up}$S genes (A), $_{up}$G and $_{up}$S genes from data excluding samples taken at subjective dusk (B), light-responsive genes in A (C), and non-light-responsive genes in A (D). Left column, $_{up}$G; right column, $_{up}$S.

tion between $_{up}$S and clock-regulated genes. These may represent targets of TFs controlled by both light and the clock, such as PIF4 and PIF5.

Genes Downstream of PIF4 and PIF5

Within the 2,000 growth-correlated genes, we were especially interested in those also regulated by *PIF4* and *PIF5*, because they are central growth regulators in our growth conditions (Nozue et al., 2007). To recover *PIF4*- and *PIF5*-regulated genes, we used Rank Product analysis (Breitling et al., 2004) to compare microarray expression profiles of wild-type Col at growing phases under SD/3 conditions with *pif4 pif5* double mutant plants at the same time (Supplemental Fig. S1). All previously identified (direct or indirect) targets of *PIF4* present on the array (*ARABIDOPSIS THALIANA HOMEOBOX PROTEIN2* [*ATHB2*], *LONG HYPOCOTYL IN FAR-RED* [*HFR1*], and *IAA29*; Lorrain et al., 2008; Koini et al., 2009) were found to be misregulated in our experiment (Supplemental Table S5). Since *PIF4* and *PIF5* are positive growth regulators, genes up-regulated by *PIF4* or *PIF5* and with expression coincident to high *PIF4* or *PIF5* protein levels during the growing phase are candidate positive growth factors operating downstream of *PIF4* or *PIF5*. Similarly, genes down-regulated by *PIF4* or *PIF5* and coincident with low *PIF4* or *PIF5* protein levels at the stationary phase are candidate growth inhibitors downstream of *PIF4* or *PIF5*. To obtain such candidates, the intersection (81 genes) of *PIF4* or *PIF5* up-regulated genes (genes with higher expression in Col than in *pif4 pif5* double mutants) and $_{up}$G genes and the intersection (39 genes) of $_{up}$S genes and *PIF4* or *PIF5* down-regulated genes were analyzed. In total, this extracted 120 genes out of 245 *PIF4*- or *PIF5*-regulated genes (Supplemental Table S5). The remaining *PIF4*- and *PIF5*-regulated genes could be involved in other *PIF4*- or *PIF5*-regulated phenomena, such as flowering time (plants overexpressing *PIF5* flower early in both long days and short days; K. Nozue and J.N. Maloof, unpublished data). In our list of 120 growth-correlated and PIF4- or PIF5-regulated genes are genes involved in three hormone-related pathways (auxin, GA, and ethylene). In addition, two genes previously implicated in growth but unconnected to hormones were found (*LONGIFOLIA1* [*LNG1*] and *LNG2*; Lee et al., 2006; Supplemental Table S4; Supplemental Fig. S2).

We next used ORA to determine if any gene categories are statistically significantly enriched in this gene list. Examining categories defined by MapMan (Supplemental Table S11A), we found that auxin metabolism genes and TF subgroups including homeobox genes (BINCODE 27.3.22, such as *ATHB2*, *-7*, *-12*, *-52*, and *HOMEOBOX FROM ARABIDOPSIS THALIANA2*) were overrepresented in $_{up}$G and *PIF4* or *PIF5* up-regulated genes (Supplemental Table S11A). Using lists of predicted hormone signaling components (Supplemental Table S4), we found that only auxin- and GA-related genes were overrepresented in $_{up}$G and *PIF4* or *PIF5* up-regulated genes (Supplemental Table S11B).

To gain a better understanding of *PIF4* and/or *PIF5* and hormone pathway interactions, ORA was done to test if any hormone-responsive genes were enriched among the *PIF4*- and/or PIF5-regulated genes (Supplemental Table S7B). We found auxin- and/or BR-responsive genes to be significantly overrepresented in *PIF4* and/or *PIF5* and growth-regulated genes. Somewhat surprisingly in the light of previous reports that PIF4 and its homolog PIF3 act in the GA pathway (de Lucas et al., 2008; Feng et al., 2008), we found only one GA-responsive gene among *PIF4*- and/or *PIF5*-regulated growth-correlated genes (Supplemental Table S7B). The discrepancy between overrepresentation of GA signaling genes and lack of enrichment of GA-responsive genes may be explained by the fact that three out of five PIF4- and/or PIF5-regulated GA "signaling" genes are GA Stimulated in Arabidopsis family members with no experimental evidence linking them to GA. In contrast to our findings with GA and consistent with a study finding that PIF4 plays an important role in plant responses to shade (Lorrain et al., 2008), we found that PIF4 and/or PIF5 up-regulated genes are enriched in shade-induced genes ($P < 0.001$; Supplemental Table S9D). Interestingly, auxin pathway genes (BINCODE 3 in Supplemental Table S4) are enriched in the set of genes regulated both by shade and by PIF4/5 (seven out of 30; $P < 0.001$).

PIF4 and PIF5 Modulate Auxin Signaling

Our bioinformatic analysis suggests that PIF4 and/or PIF5 may regulate growth via the control of auxin signaling. To test this experimentally, we examined hypocotyl response to auxin in plants overexpressing *PIF5* (*PIF5-OX*) and in *pif4 pif5* double mutants. In these lines, responses to natural (IAA) and synthetic (picloram) auxins were perturbed in several ways when compared with the wild type (Fig. 3, A–D). First, the growth inhibition response to high auxin concentrations is altered; *pif4 pif5* mutants are less sensitive to auxin for this response, and plants overexpessing *PIF5* are more sensitive. Examining growth promotion relative to untreated controls (Fig. 3, B and D), *pif4 pif5* are more responsive than the wild type, whereas *PIF5* overexpressors are less responsive. These results are consistent with the idea that the *pif4 pif5* growth defect arises in part due to altered auxin levels, although, as discussed below, altered auxin levels cannot explain the entire phenotype.

Another way to examine auxin responsiveness is to grow plants at elevated temperatures, which causes increased levels of bioactive auxin and stimulates hypocotyl growth (Gray et al., 1998). Therefore, we examined the effects of temperature on hypocotyl elongation of *PIF5-OX* and *pif4 pif5* (Fig. 4). The *pif4 pif5* mutant remained short even under high temper-

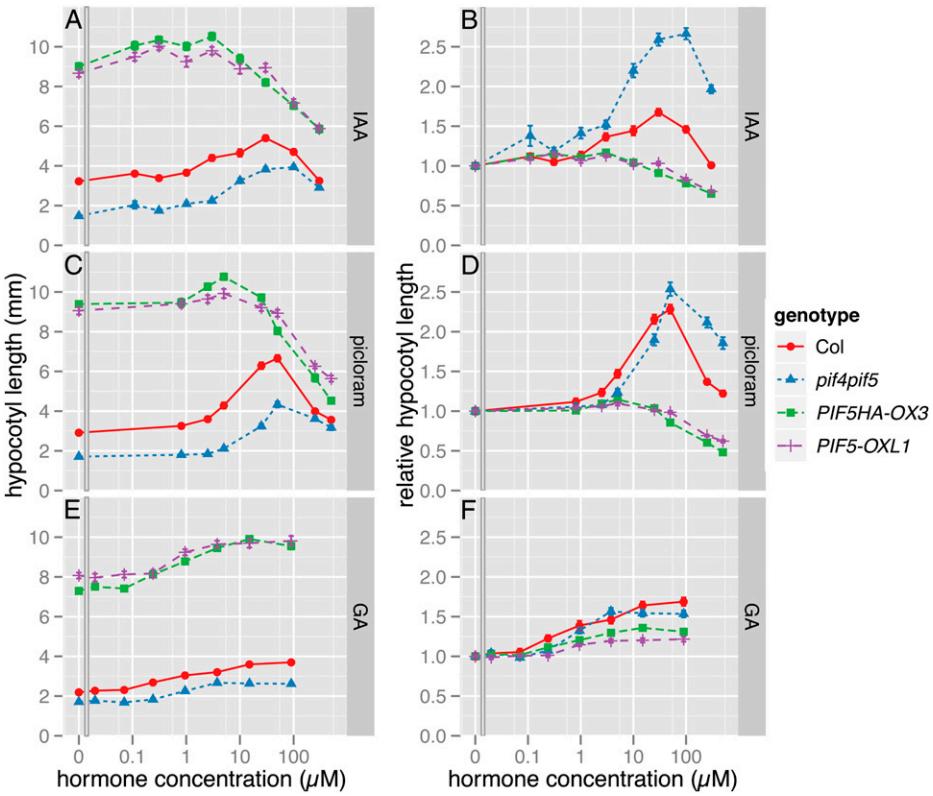

Figure 3. PIF4 and/or PIF5 affect plant sensitivity to auxin more than to GA. Plants were grown in short days for 3 d and then transferred to plates containing the indicated hormones. After an additional 7 d of growth, seedling height was measured. Dose-response curves are shown for hypocotyl length in response to treatment with IAA (A and B), picloram (C and D), or GA (E and F). B, D, and F show normalized responses; for each genotype, the values were divided by the average hypocotyl length of that genotype without added hormone. Both *PIF5-HA-OX3* and *PIF5-OXL1* overexpress *PIF5*. Error bars show SE; data from at least two independent experiments are shown; $166 \leq n \leq 264$ seedlings. [See online article for color version of this figure.]

ature (i.e. less responsive to high temperature). Responsiveness of *PIF5-OX* plants to temperature is also reduced, due to the elongated hypocotyls of these plants at ambient temperatures (Fig. 4), These results are consistent with recent reports that the *pif4* single mutant and the *pif4 pif5* double mutant show reduced responses to high temperature (Koini et al., 2009; Stavang et al., 2009). In combination with the altered responsiveness of *pif4 pif5* plants to exogenous auxin (Fig. 3), this result indicates that *PIF4* and/or *PIF5* modulate auxin pathways.

Because we found that PIF4 and/or PIF5 up-regulate expression of the auxin biosynthetic gene *YUCCA8*, it is possible that overproduction of auxin is the primary mode of action for *PIF5-OX*. To test this idea, we measured hypocotyl growth rate of *yucca*, an auxin-overproducing mutant (Zhao et al., 2001; Fig. 5A). Overall, *yucca* hypocotyl showed altered growth rhythms compared with the wild type, consistent with our prediction of auxin signaling as important to rhythmic growth. However, *yucca* still had a diurnal growth pattern with peaks at dawn, similar to wild-type plants, and did not show the immediate growth response to darkness seen in *PIF4-OX* and *PIF5-OX* (Nozue et al., 2007). In contrast, the baseline growth rate in *yucca* mutants is higher than in the wild type, similar to that of *PIF5-OX* (Nozue et al., 2007). To analyze growth pattern objectively, we applied network analysis to quantitative time-course growth rate data based on similarities of growth patterns (Fig. 5B). The growth rate network analysis clearly demonstrated distinctive subgroups consisting of the wild type and arrhythmic clock mutants. As we expected from comparison of the raw data, the *yucca* growth pattern was correlated with both the wild type and *PIF5-OX* but showed a stronger connection to the wild type. This suggests that the *PIF5-OX* growth phenotype cannot be explained solely by constitutive auxin production.

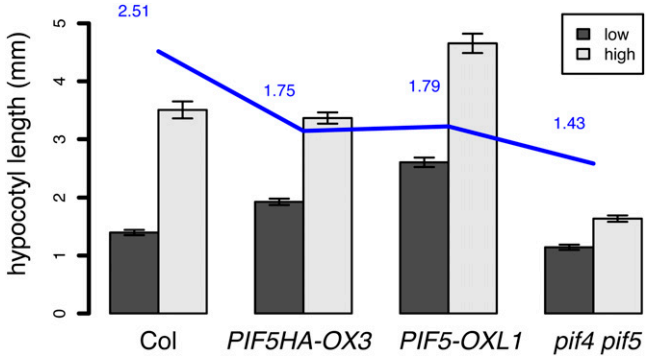

Figure 4. Promotion of growth by high temperature is impaired in *pif4 pif5*. Hypocotyl lengths at low temperature (black bars) and at high temperature (gray bars) are shown. Ratios of hypocotyl length (high temperature to low temperature) are shown by the blue line and numbers. Error bars show SE; data from two independent experiments are shown; $47 \leq n \leq 61$ seedlings. [See online article for color version of this figure.]

To further assay the effect of *PIF4* and/or *PIF5* on auxin-related processes, we examined polar auxin transport. In our microarray data, we found that *PIF4* and/or *PIF5* up-regulate some polar auxin transport-related genes such as *PIN-FORMED3* (Friml et al., 2002), *ATHB2* (Steindler et al., 1999), and *WAG2* (Santner and Watson, 2006; Supplemental Table S5; Supplemental Fig. S2). To test whether *PIF4* and/or *PIF5* might affect transport-related processes, we examined the effects of *PIF5-OX* and *pif4 pif5* on hypocotyl elongation in the presence of the polar auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA; Supplemental Fig. S3). Compared with the wild type, *pif4 pif5* shows significantly reduced responses to NPA at all concentrations tested. *PIF5-OX* does respond to NPA but requires concentrations three times higher than the wild type to show a significant response, and the relative response of *PIF5-OX* is reduced at NPA concentrations below 1 μM. These data are consistent with PIF4 and PIF5 modulating auxin-related processes.

GA Biosynthesis Contributes to Rhythmic Growth But Is Separable from PIF4/5 Pathways

Since ORA of hormone signaling genes (Supplemental Table S6) and hormone-responsive genes (Supplemental Table S7) predict the involvement of GA pathways in rhythmic growth, we tested whether reduced GA biosynthesis could result in retarded rhythmic growth. We examined plants mutant for *GIBBERELLIN 20-OXIDASE1* (*GA20OX1*) and *GA20OX2*, which encode key enzymes in the biosynthesis of gibberellins. *GA20OX1* and *GA20OX2* are $_{up}$G genes, and it has been shown that the *ga20ox1 ga20ox2* mutant germinates normally but has short hypocotyls (Rieu et al., 2008). Indeed, we found that the double mutant clearly has a growth defect during the late night (Fig. 6). We next asked if GA signaling is modulated by *PIF4* or *PIF5*. Compared with auxin, altered *PIF4* or *PIF5* expression had less effect on growth responses to exogenous GA (Fig. 3, E and F). The magnitude of the effects seen here is similar to that reported by de Lucas et al. (2008), where exogenous GA caused a 60% increase in wild-type elongation and where the absolute increase in hypocotyl length of *pif4* (de Lucas et al., 2008) or *pif4 pif5* (this study) was only about 50% of the wild type. It is interesting that *pif4 pif5* is very similar to the wild type when the GA response is normalized to untreated controls (Fig. 3F), a phenomenon that is also apparent in the de Lucas et al. (2008) data (data not shown). Overall, these data suggests that in our growth conditions, *PIF4* and/or *PIF5* exert larger effects on auxin signaling than on GA signaling, consistent with the lack of enrichment of GA-responsive genes in *PIF4* and/or *PIF5* growth-correlated genes (Supplemental Table S7B). These results suggest that although GA biosynthesis is required for normal growth patterns, under the growth conditions used here, PIF4 and/or PIF5 are not important regulators of the GA pathway.

DISCUSSION

Global Interactions between the Circadian Clock and Light

Our analysis of the frequency distribution of the circadian time of peak expression in light-responsive genes suggests that, globally, circadian expression of light-induced genes anticipates dawn and expression of light-repressed genes anticipates dusk (Fig. 1). The dual regulation by light signaling and the clock should ensure proper expression patterns to adapt to predictable diurnal fluctuations. One example is the expression of photosynthesis genes (Table II). They are clock regulated with peak expression in the morning, likely because they are required every day for proper photosynthesis; they are also induced by light, perhaps to allow for day-to-day variation in light intensity.

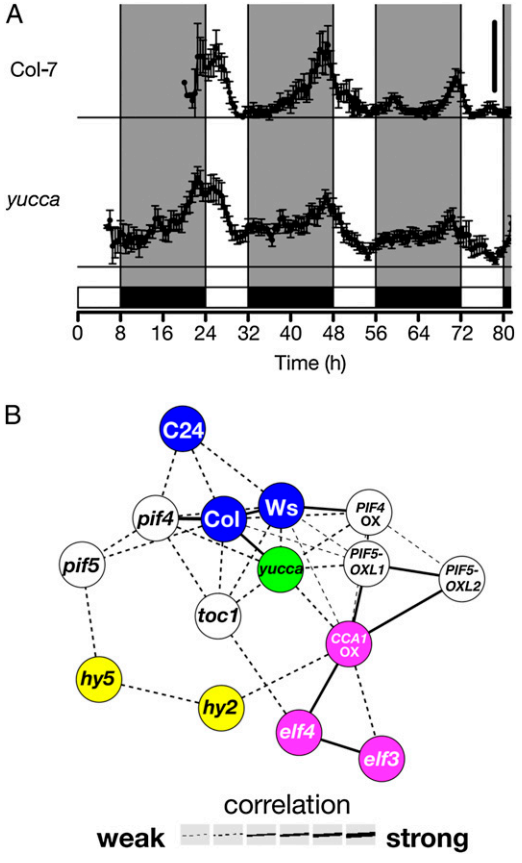

Figure 5. Auxin overproduction partially disrupts growth rhythms. A, Growth kinetics of auxin-overproducing *yucca* seedlings. Col-7 is the wild-type background for *yucca*. Times of light and darkness are indicated by white and black rectangles on the *x* axis and by white and gray areas on the plot. B, Network analysis of growth kinetics. Each node represents a genotype, and each edge indicates the similarity between their growth patterns. Degree of similarity is indicated from dotted (lower) to thick (higher) lines. Blue, yellow, magenta, and green node colors indicate wild-type, light signaling, arrhythmic clock, and auxin overproduction plants, respectively.

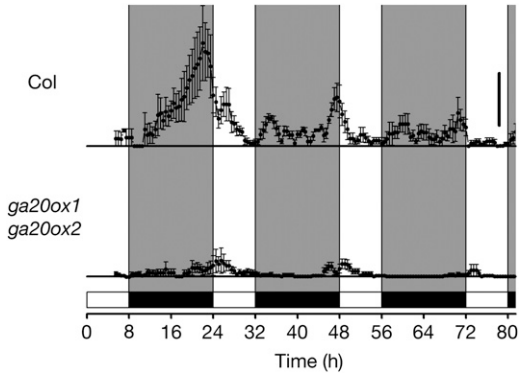

Figure 6. Reduction of active GA abolishes rhythmic growth. Col is the wild-type background for the *ga20ox1 ga20ox2* double mutant. The experiment was performed as described for Figure 5A.

It has been demonstrated that the clock temporally modulates plant sensitivity to light (a process called gating). So far, this gating has only been shown for a few genes (*CHLOROPHYLL A/B BINDING PROTEIN2* [Millar and Kay, 1996], *PHYTOCHROME INTERACTING FACTOR3-LIKE1* [Salter et al., 2003], and *GIGANTEA* [Paltiel et al., 2006]), which are all light induced. However, we have identified both many light-induced genes with dawn-phased clock regulation and many light-repressed genes with dusk-phased clock regulation (Fig. 1), suggesting that gating of both light-induced and light-repressed genes may be extensive. This analysis suggests (1) that gating of light-responsive genes is a genome-wide phenomenon and (2) that gating is as prevalent for light-repressed genes as for those that are light induced.

Global Interactions between Light and Growth

Light and Auxin

Extensive cross talk occurs between the light and auxin signaling pathways, with light affecting auxin biosynthesis, auxin transport, and auxin-responsive gene expression (Sibout et al., 2006; Laxmi et al., 2008; Tao et al., 2008). Since light induces the rapid degradation of PIF4 and PIF5 and we found that they in turn modulate auxin sensitivity, it is likely that another interaction between the light and auxin pathways occurs through *PIF4* and *PIF5*. Two bZIP TFs, *LONG HYPOCOTYL5* (*HY5*) and *HY5 HOMOLOG* (*HYH*), integrate light signals from phytochromes and cryptochromes to repress hypocotyl growth (Li and Yang, 2007; Bae and Choi, 2008). *HY5* and *HYH* also modulate auxin sensitivity, possibly by regulating the expression of auxin pathway genes (Sibout et al., 2006). There is a statistically significant overlap between genes regulated by PIF4 and/or PIF5 and those regulated by HY5 and HYH (Sibout et al., 2006; Supplemental Results S1; Supplemental Table S13), suggesting that the *HY5 HYH* and *PIF4 PIF5* downstream networks may overlap. Another link between light and auxin via *PIF4* or *PIF5* could be the regulation of auxin transport. Phytochrome is known to change the rate of auxin transport (Salisbury et al., 2007). Our data showed that altered *PIF4* and/or *PIF5* expression resulted in resistance to a polar auxin transport inhibitor (Supplemental Fig. S3), supporting this link.

Our data analysis, which showed the lack of enrichment of auxin-related genes in light-responsive clock-regulated genes, suggests that auxin pathways are regulated differently between light and clock. This suggested that transcriptional regulation of auxin pathways by light differs from regulation by the clock, even though many auxin-responsive genes overlap with both light-responsive genes and clock-regulated genes. Consistent with this idea, there is overlap between the auxin pathway genes regulated by light and *PIF4* and *PIF5*, but not between those regulated by the clock and *PIF4* and *PIF5* (BINCODE 2 in Supplemental Table S4). Fundamentally, *PIF4* and *PIF5* are light-response genes whose activity is gated by the clock.

Shade

Our data showed that *PIF4*- or *PIF5*-regulated genes, growth-correlated genes, and shade-responsive genes significantly overlapped. Auxin pathway genes are enriched in the overlapping genes, consistent with the recent finding that shade-induced auxin biosynthesis is involved in shade-induced growth promotion (Tao et al., 2008). This suggests that the overlapping auxin pathways represent a shared growth control mechanism between *PIF4* or *PIF5* and shade-promoted growth. In addition, *PIF4* or *PIF5* is required for shade-induced growth (Lorrain et al., 2008), supporting our hypothesis.

PIF5 Modulates Auxin Pathways

Our data clearly show that *PIF5* (and possibly *PIF4*) modulates auxin sensitivity because (1) many auxin pathway genes are regulated by *PIF4* or *PIF5*, (2) *PIF5-OX* and *pif4 pif5* show altered sensitivity to exogenous auxin, and (3) *pif4 pif5* is impaired in auxin-mediated growth promotion at elevated temperatures. However, growth control by *PIF4* or *PIF5* is not simply via the control of auxin levels, because *PIF5-OX* plants show greater growth deregulation than seen in the auxin-overproducing *yucca* plants. This conclusion is supported by the observation that treatment of *pif4 pif5* mutants with exogenous auxin does not rescue their short-hypocotyl phenotype (Fig. 4), in contrast to the rescue seen in auxin-deficient *rve1* mutants (Rawat et al., 2009).

If PIF4 and/or PIF5 are not regulating auxin levels, how do they modulate the auxin pathway? Many auxin pathway genes are regulated by *PIF4* or *PIF5*, suggesting that they may transcriptionally regulate one or more master regulators of auxin sensitivity.

Possible candidates include *MINI ZINC FINGER1* (Hu and Ma, 2006), *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*; Hu et al., 2003), *ARGOS-LIKE* (Hu et al., 2006), and *HFR1* (Sessa et al., 2005; Hornitschek et al., 2009), genes with expression regulated by *PIF4* and/or *PIF5* (Supplemental Table S5) and that are known to affect auxin responses or the induction of auxin-responsive gene expression. In one sense, *HFR1* is a strong candidate, because PIF5 binds to G-boxes in the *HFR1* promoter region in vivo (Hornitschek et al., 2009). On the other hand, since HFR1 has an atypical bHLH domain, it is unclear if it directly regulates auxin-responsive genes.

Polar auxin transport-related proteins are likely also involved in the regulation of auxin response by *PIF4* and/or *PIF5* (see "Light and Auxin" above). To explain why both *PIF5-OX* and *pif4 pif5* show NPA insensitivity in addition to altered auxin responses, we propose a simple model (Fig. 7). Typical of many hormone response curves, in this model there is a log-linear response range; plants show relatively little response to auxin below this range and show growth inhibition above it. We suggest that without added auxin or NPA, wild-type plants are roughly in the middle of the linear response range, *PIF5* overexpression shifts plants to near their maximum response, and knockout of *pif4* and/or *pif5* shifts plants to near the bottom of the log-linear range. Because of these shifts, exogenous auxin decreases hypocotyl elongation at a lower concentration in *PIF5-OX* than in the wild type; in contrast, *pif4 pif5* requires a higher concentration of auxin to stimulate hypocotyl elongation. Since NPA blocks polar auxin transport from the shoot apex, NPA treatment should lead to decreased levels of endogenous auxin in growing hypocotyl tissue. The reduced sensitivity of both *PIF5-OX* and *pif4 pif5* to NPA might be caused by both genotypes being outside the log-linear response range for auxin modulation of hypocotyl growth.

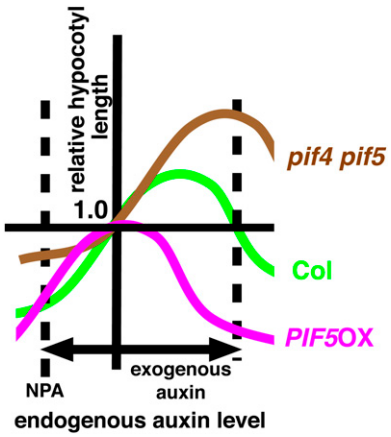

Figure 7. A model of *PIF4*- and/or *PIF5*-regulated auxin responses. In wild-type plants, increasing auxin levels up to an optimal concentration lead to longer hypocotyls; auxin levels higher than this cause growth inhibition. Overexpression of *PIF5* sensitizes, while loss of *PIF4* and/or *PIF5* desensitizes, hypocotyl responses to auxin. We assume that NPA treatment reduces endogenous auxin in the hypocotyl. Note that this model fits all dose-response curves in Figure 3, A and B, and Supplemental Figure S3. [See online article for color version of this figure.]

Links between PIF4, PIF5, and GA Signaling

In our experimental conditions, alteration of *PIF5* transcript level had noticeably less effect on GA response that it did on auxin response (Fig. 3). This suggests that PIF5 is not an important regulator of plant responses to GA in our assays. Consistent with this, we found no significant overlap between GA-responsive genes and *PIF4*- or *PIF5*-regulated genes (Supplemental Table S7). This is quite different from previous reports, in which PIF4 and PIF5 as well as PIF3, a close homolog, were found to modulate GA signaling through interaction with DELLA proteins (de Lucas et al., 2008; Feng et al., 2008). These discrepancies may be due to differences in light conditions used in these experiments; light promotes PIF protein degradation but stabilizes DELLA proteins (Achard et al., 2007; Castillon et al., 2007), meaning that the window of opportunity for interaction may be limited depending on the light conditions. In this study, we grew plants in short days with 54.9 μE white light, whereas de Lucas et al. (2008) grew plants in 35 μE continuous red light. The effect of light conditions on interactions between the DELLAs and PIF5 should be tested in the future.

Do PIF4 and/or PIF5 Function through Phytochrome B to Promote Growth?

It has been proposed that growth promotion by *PIF5-OX* under continuous red light is mediated by reduced phytochrome B (phyB) protein level (Khanna et al., 2007). Recently, *PIF3* was proposed to have dual functionality that uses transcriptional activity for early responses and regulation of phyB abundance to mediate long-term growth inhibition (no *PIF3* transcription activity required; Al-Sady et al., 2008). It is interesting to ask whether *PIF4* and *PIF5*, close homologs of *PIF3*, function in a similar manner, and specifically whether this model can explain *PIF4* or *PIF5* activities in promoting daily growth rhythms. Although phyB protein abundance drops significantly when etiolated seedlings are exposed to light, these changes are slower than the growth responses we observed in light-grown plants under short days or SD/3 (Nozue et al., 2007; Leivar et al., 2008; Rausenberger et al., 2010). More relevant to our studies in light-grown plants is the finding that phyB abundance does not change dramatically over the course of a day under day/night cycles (Sharrock and Clack, 2002). Thus, it is unlikely that under SD/3 conditions, dark-induced rapid growth is mediated by a decreased level of phyB protein. This suggests that *PIF4*- or *PIF5*-

regulated daily growth is mediated by their transcription activities.

Pathways Predicted to Generate Rhythmic Growth

Our data predict that diurnal regulation of the auxin, GA, BR, and possibly ethylene pathways is important for rhythmic hypocotyl growth, consistent with a recent study (Michael et al., 2008). PIF4 and/or PIF5 both control diurnal growth rhythms (Nozue et al., 2007) and modulate plant sensitivity to auxin (Fig. 3), suggesting that their control of daily growth is mediated in part via their regulation of the auxin pathway. Diurnal regulation of *GA20OX1* and *GA20OX2* transcripts, which peak during the growing phase, may also be important for daily growth rhythms (Fig. 6). However, because in our conditions genetic manipulation of *PIF4* and *PIF5* had little effect on GA responsiveness (Fig. 3), our data suggest that *GA20OX1* and *GA20OX2* control of growth is likely separable from *PIF4* and/or *PIF5* growth control.

Our analysis predicts that genes that encode some classes of cell wall-modifying enzymes and wax biosynthesis genes (Supplemental Results S1) are involved in rhythmic growth. Expression of some of these genes is regulated directly or indirectly by *PIF4* and/or *PIF5*. During the stationary phase, wax and lignin biosynthesis genes are up-regulated. If enzyme activities of these genes correspond to their mRNA patterns, then this temporal regulation could indicate that the stationary phase is the best time for adding strengthening and protective materials to the cell wall and plant surface. There is currently no information on their function in hypocotyl growth, suggesting that further studies may be fruitful.

A full understanding of growth-control networks will require many types of physiological and molecular data. Currently, quantitative time-course data are available for only transcript levels and hypocotyl growth rates. Analysis of transcript levels is not sufficient to obtain the entire picture of growth-control systems. For example, *PIF5* is not found in our list of genes with growth-correlated expression, despite the key role it plays in growth regulation. This discrepancy is likely because PIF4 and PIF5 protein levels are rapidly reduced in response to light (Nozue et al., 2007), suggesting that the abundance of PIF4 and PIF5 proteins likely does correlate with growth. In the future, combining the systems biology approaches used here with multilayered time-course data, including protein abundance, protein-protein interactions, metabolite level, and enzyme activities, should further untangle the webs of growth-control systems.

Our association analysis of transcript levels with growth has shown that only one-twelfth of all Arabidopsis genes have an expression pattern correlated with growth. Therefore, such studies of temporal changes in plant physiology serve as efficient tools for narrowing the number of candidate genes that regulate plant growth and dynamically control cell wall modification.

MATERIALS AND METHODS

Plant Materials

Seeds of Arabidopsis (*Arabidopsis thaliana*) *PIF5-OXL1*, *PIF5-OXL2*, *PIF5-HA-OX3*, and *pif4 pif5* were described previously (Fujimori et al., 2004; Nozue et al., 2007; Lorrain et al., 2008). Seeds of *yucca* (Zhao et al., 2001) were kindly provided by Joanne Chory (Salk Institute for Biological Studies). *ga20ox1 ga20ox2* (Rieu et al., 2008) seeds were kindly provided by Peter Hedden (Rothamsted Research). *CCA1-OX* seeds (Wang and Tobin, 1998) were kindly provided by Elaine Tobin (University of California, Los Angeles). Culture methods for hypocotyl growth rate measurements are described (Nozue et al., 2007).

For exogenous hormone treatment, seeds were surface sterilized with 70% ethanol/0.1% Triton X-100 for 5 min followed by 95% ethanol for 1 min. Sterilized seeds were resuspended in sterile water and plated on 15 mL of half-strength Murashige and Skoog basal salts with minimal organics (MSMO; M6899; Sigma) and 0.8% agar (A1296; Sigma). Seeds were stratified at 4°C for 4 d and incubated under short-day conditions at 22°C (8 h of white fluorescent light, 16 h of dark) for 3 d. Germinated seeds were transferred to half-strength MSMO agar plates containing hormone concentration series (for IAA, 0, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μM; for picloram, 0, 0.8, 2.5, 5, 25, 50, 100, and 250 μM; for GA_3, 0, 0.01, 0.06, 0.23, 0.94, 3.75, 15, and 60 μM) and incubated under short-day conditions for 7 d. To prevent degradation of IAA, long-pass light filters (Yellow Plexiglas 2208 acrylic sheet; Ridout Plastics; Stasinopoulos and Hangarter, 1990) were used to cover plates, resulting in yellow light with a fluence rate of 54.9 μE. The same condition (including the filter) was used for exogenous GA treatment. For NPA treatment, sterile seeds were placed on half-strength MSMO plates containing an NPA concentration series (0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM) and the plants were grown under short days for 7 d under fluorescence lamps with a fluence rate of 50.2 μE. For high-temperature treatment, plates with 3-d-old germinated seeds were transferred to a chamber under the high-temperature condition (28°C) or kept in the same chamber. During the temperature experiment, the short-day condition was unchanged. Seedling height was measured 7 d after transfer to the high-temperature environment.

For RNA extraction, seedlings grown under short-day conditions for 3 d were transferred to SD/3 conditions (160-min-light/320-min-dark cycles) from the dawn of day 4 as described (Nozue et al., 2007). Sample collection time is summarized in Supplemental Figure S1. Briefly, seedlings of Col and *CCA1-OX* were collected at 120 min (light), 280 min (dark), 1,080 min (light), 1,240 min (dark), 1,560 min (light), 1,720 min (dark), 2,520 min (light), 2,680 min (dark), 3,000 min (light), 3,160 min (dark), 3,960 min (light), and 4,120 min (dark). Independent samplings were repeated once except for three time points (2,520, 3,000, and 4,120 min). Seedlings of *pif4 pif5* were independently collected in duplicate at 280, 1,720, and 3,160 min, corresponding to times when hypocotyl growth occurs in Col but not in *pif4 pif5*. RNA extraction and microarray experiments were performed as described previously (Nozue et al., 2007). Recurrent time points (every 1,440 min per 24 h) were used as biological replicates in the subsequent analysis.

Genomic Analysis

Most analyses were done using R (R Development Core Team, 2005 [http://www.R-project.org/]) and Bioconductor (Gentleman et al., 2004 [http://www.bioconductor.org]). Gene annotations were based on the TAIR8 version of the Arabidopsis genome (http://www.arabidopsis.org/) and published literature. Rank Product analysis was done by the RankProd package in R with a FDR of less than 0.01 and fold change of less than 0.9 (for $_{up}$G or $_{up}$G without dusk samples) or greater than 1.1 (for $_{up}$S or $_{up}$S without dusk samples; Breitling et al., 2004; Hong et al., 2006). Our complete time-course microarray data (see Results) have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE21684. Visualization (Fig. 2) of $_{up}$G or $_{up}$S genes found in known growth-controlling pathways was done using MapMan software with a custom pathway map (Supplemental Fig. S2) and custom mapping data (Supplemental Table S4). ORA was mainly performed using the PageMan Web application and the MapMan gene ontogeny (the gene ontog-

eny used in the MapMan software [http://mapman.mpimp-golm.mpg.de/general/ora/ora.shtml]; Usadel et al., 2006) with TAIR8 as a control group. Since the MapMan ontogeny does not have comprehensive hormone pathway categories, we chose the hormone genes (Supplemental Table S4) based on the literature and used these for MapMan analysis and ORA by Fisher's exact test (Supplemental Tables S7A and S11B). For the same reason, genes listed on the Purdue University Cell Wall Genomics Web page (http://cellwall.genomics.purdue.edu/families/index.html) were used for ORA of genes encoding cell wall biosynthesis or modification enzymes. A set of Gene Expression Omnibus microarray data (accession no. GSE5617; Peschke and Kretsch, 2011) was used for extracting light-responsive genes by Rank Product analysis with a FDR of less than 0.01. Hormone-responsive genes were those defined in Supplemental Table S9 in Nemhauser et al. (2006) and Supplemental Table S1 in Zentella et al. (2007). Shade-responsive genes were those defined in Supplemental Table S4 in Carabelli et al. (2007).

Time-Lapse Photography and Image and Network Analysis

These analyses were performed as described previously (Nozue et al., 2007). In addition to R and Bioconductor, Cytoscape (Shannon et al., 2003 [http://www.cytoscape.org/]) and Gaggle (Shannon et al., 2006 [http://gaggle.systemsbiology.net/docs/]) were used for growth phenotype network analysis.

Microarray data from this article can be found in the Gene Expression Omnibus database under accession number GSE21684.

Supplemental Data

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

Supplemental Figure S1. Sampling schedule for the microarray experiment.

Supplemental Figure S2. MapMan analysis of known growth-controlling genes.

Supplemental Figure S3. *PIF4* and/or *PIF5* affect plant sensitivity to polar auxin transport inhibitor.

Supplemental Table S1. List of light-responsive genes.

Supplemental Table S2. Clock, light, and growth interactions revealed in whole-genome expression patterns.

Supplemental Table S3. ORA of light-responsive and clock-regulated genes.

Supplemental Table S4. Known growth-controlling genes.

Supplemental Table S5. A complete list of growth-correlated genes.

Supplemental Table S6. ORA of growth-correlated genes with hormone genes, cell wall biosynthesis/modification genes, and ABC transporters

Supplemental Table S7. ORA of hormone-responsive genes in growth-correlated genes.

Supplemental Table S8. ORA of light-responsive and growth-correlated genes.

Supplemental Table S9. Correlations among shade-responsive genes, light-responsive genes, and growth-correlated genes.

Supplemental Table S10. ORA of clock-regulated and growth-correlated genes.

Supplemental Table S11. ORA analysis of *PIF4*- and/or *PIF5*-regulated and growth-correlated genes.

Supplemental Table S12. Fisher's exact test for interaction between growth-correlated genes and *HY5 HYH*-regulated genes.

Supplemental Table S13. Fisher's exact test for interaction between *PIF4* and/or *PIF5* growth genes and *HY5 HYH*-regulated genes.

Supplemental Table S14. Fisher's exact test for interaction between growth-correlated genes and *SIZ1*-regulated genes.

Supplemental Table S15. ORA for interaction between *PIF4* and/or *PIF5* growth genes and *SIZ1*-regulated genes.

Supplemental Results S1. Additional results and discussion with more details on candidate and known growth genes found in this study, comparing the growth network defined here with those found in other studies.

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