Circadian oscillation of gibberellin signaling in *Arabidopsis*

María Verónica Arana[a,1], Nora Marín-de la Rosa[a], Julin N. Maloof[b], Miguel A. Blázquez[a,2], and David Alabadí[a]

[a]Institute of Molecular and Plant Cellular Biology, Consejo Superior de Investigaciones Científicas–Universidad Politécnica de Valencia, 46022 Valencia, Spain; and [b]Department of Plant Biology, University of California, Davis, CA 95616

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Circadian clocks are endogenous timekeeping mechanisms that allow organisms to anticipate rhythmic, daily environmental changes. Temporal coordination of transcription results in a set of gene expression patterns with peak levels occurring at precise times of the day. An intriguing question is how a single clock can generate different oscillatory rhythms, and it has been proposed that hormone signaling might act in plants as a relay mechanism to modulate the amplitude and the phase of output rhythms. Here we show that the circadian clock gates gibberellin (GA) signaling through transcriptional regulation of the GA receptors, resulting in higher stability of DELLA proteins during daytime and higher GA sensitivity at night. Oscillation of GA signaling appears to be particularly critical for rhythmic growth, given that constitutive expression of the GA receptor expands the daily growth period in seedlings, and complete loss of DELLA function causes continuous, arrhythmic hypocotyl growth. Moreover, transcriptomic analysis of a pentuple *della* KO mutant indicates that the GA pathway mediates the rhythmic expression of many clock-regulated genes related to biotic and abiotic stress responses and cell wall modification. Thus, gating of GA sensitivity by the circadian clock represents an additional layer of regulation that might provide extra robustness to the diurnal growth rhythm and constitute a regulatory module that coordinates the circadian clock with additional endogenous and environmental signals.

crosstalk | plasticity | light | photoperiod

The pervasive role of the circadian clock driving plant physiology is reflected by the extensive regulation it exerts on gene expression, as more than one third of *Arabidopsis* genes are under circadian control (1). Remarkably, the expression of almost every single gene of *Arabidopsis* cycles when plants are grown under more realistic situations, for example combinations of thermo- and photocycles (2). This suggests that the entrainment of the circadian clock by light and temperature signals might allow plants to adapt to the daily changes in the environment by timing every physiological pathway to the specific time of day when it is more advantageous. For instance, the concerted action of the circadian clock and phyB-mediated light signaling allows the expression of a set of hormone-related genes toward dawn (3), which may provide robustness to the rhythmic patterns of growth of the seedling under diurnal conditions (4). Accordingly, a correlation exists between the oscillation of auxin-related genes and changes in the hypocotyl growth rate of seedlings grown under free-running conditions (5, 6), although the physiological significance of this correlation remains to be explored. In addition to light-mediated growth, the circadian clock controls the time of day that other environmental response pathways can be activated, often by triggering the oscillation of key signaling genes involved in these pathways (7). This type of regulation is known as gating because the clock can be thought of as opening or closing a gate to control the flow of information through a signaling pathway. Through such gating the circadian clock regulates many physiological responses including the photoperiodic induction of flowering and stress responses (8).

The phytohormone gibberellin (GA) has a prominent role in the regulation of several developmental programs also affected by light and the circadian clock, including the establishment of photomorphogenesis (9–11) and cell expansion (12), and the question arises of whether GA activity might mediate circadian regulation of clock targets. Given that such a regulatory mechanism would impact the robustness and flexibility of circadian regulation of development, we decided to explore this possibility and its physiological relevance.

Results and Discussion

Expression of GA Receptors Is Controlled by the Circadian Clock. To investigate whether the circadian clock regulates GA signaling in *Arabidopsis*, we examined the daily expression pattern of all known GA signaling elements in the DIURNAL database (http://diurnal.cgrb.oregonstate.edu/) (2, 13). Although a weak oscillation could be detected in some cases, mostly linked to temperature rhythms, only the *GID1* receptor genes displayed robust cycling under short days (Fig. S1*A*). The cycling of *GID1a* and *GID1b* was validated by real-time quantitative RT-PCR in independent time-course experiments, whereas we were not able detect oscillation for *GID1c* transcript (Fig. 1*A*). The anticipation of changes in transcript levels to the light-to-dark and dark-to-light transitions and the oscillation under continuous light in entrained seedlings (Fig. S1*B*) suggested circadian rather than diurnal regulation. This was confirmed by analyzing mRNA levels in mutants defective for clock function, *toc1-1* (14) and *lhy* (15). The waveform of the oscillation in *toc1-1* seedlings was different from the WT, and the peak was narrower and was phased earlier (Fig. 1*F*). The phase advance is typical of *toc1-1* mutants, as a result of the deviation between their endogenous period (21 h) and the length of the day (24 h) (16). Transcript levels of both genes were altered also in the arrhythmic mutant *lhy* (Fig. 1*K*).

The expression of GA receptor genes is known to respond to endogenous GA levels through a DELLA-mediated feedback mechanism, i.e., their expression increases when GA levels are low and decreases when hormone levels are high (17). Hence, oscillation of *GID1* transcript levels might be a direct consequence of the circadian clock activity or, alternatively, it might respond to a putative oscillation of GA levels. However, *GID1a* expression was not altered in seedlings of the quadruple *della* mutant (*rga-t2 gai-t6 rgl1-1 rgl2-1*) (18, 19) (Fig. S2), indicating

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[1]Present address: Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Bariloche and Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, 8400 Bariloche, Río Negro, Argentina.

[2]To whom correspondence should be addressed. E-mail: mblazquez@ibmcp.upv.es.

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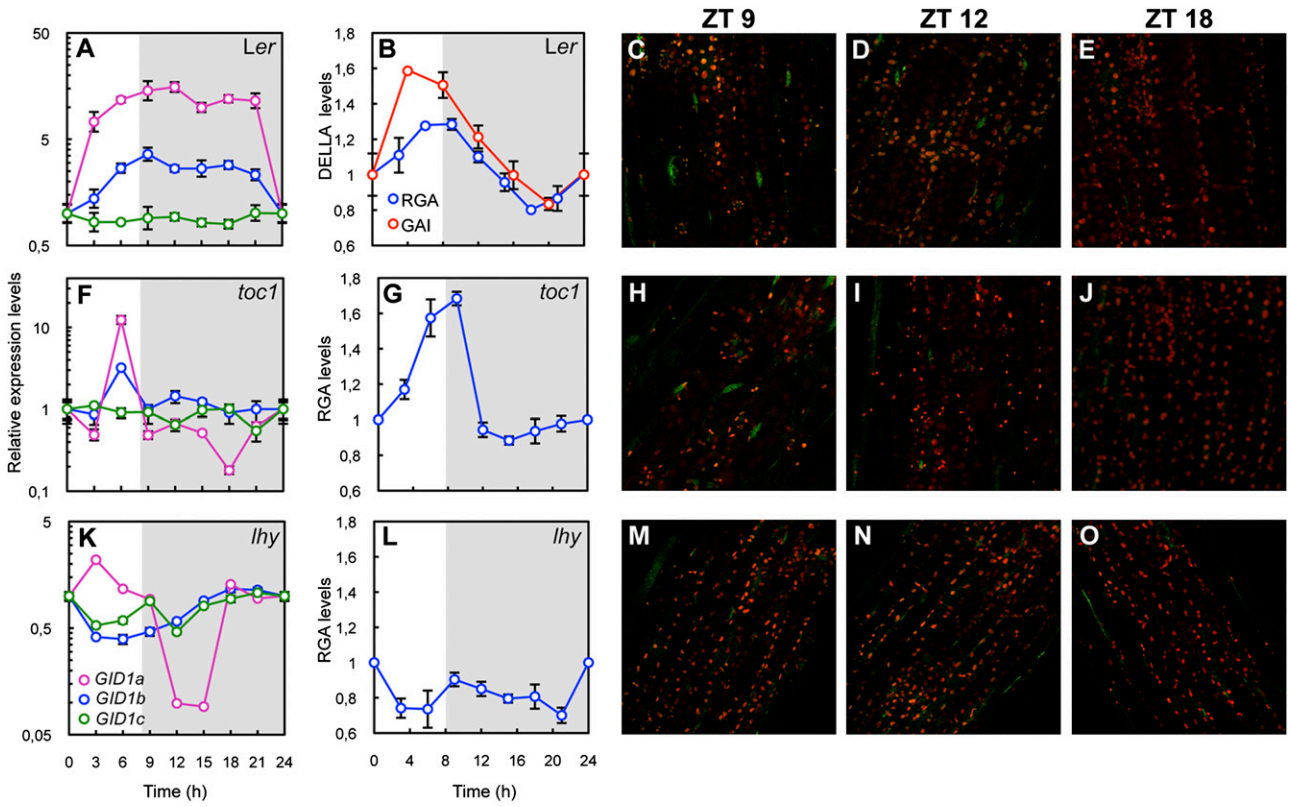

Fig. 1. The circadian clock controls the diurnal oscillation of DELLA proteins in the cell expansion zone of hypocotyls. Expression of *GID1a*, *GID1b*, and *GID1c* in 5-d-old L*er* WT seedlings (*A*), in *toc1-1* (*F*), and in *lhy* (*K*) mutants grown under short-day photocycles (8 h light/16 h dark). Values are expressed relative to *PP2a* expression. In *B–E*, *G–J*, and *L–O*, seedlings carrying the *35S::TAP-GAI* or *RGA::GFP-RGA* constructs were grown for 5 d under short-day photocycles (8 h light/16 h dark). DELLA protein levels in the L*er* WT (*B*) and in the *toc1-1* (*G*) and *lhy* (*J*) mutants were determined by Western blot analysis. TAP-GAI and GFP-RGA proteins were detected with commercial antibodies against the *myc* tag and GFP, respectively. DELLA levels were normalized against levels of DET3, which was used as loading control. Data are average of three independent experiments and plotted as mean ± SEM. Protein level at ZT0 was set to 1 and used as reference for all other time points. White and gray areas represent day and night, respectively. Fluorescence of GFP-RGA oscillates in the upper part of hypocotyls of L*er* WT (*C–E*) and *toc1-1* mutant seedlings (*H–J*), but not in the *lhy* mutant (*M–O*). Fluorescence was detected by confocal microscopy. Images are representative of three independent biological repeats including 12 to 15 seedlings per time point and per genotype.

that the circadian clock controls the expression of *GID1* genes independently of the status of the GA pathway.

Levels of DELLA Proteins Oscillate with a Daily Rhythm. GID1 receptors are known to interact with DELLA proteins in a GA-dependent way and promote their degradation (17). Thus, if the oscillation of GID1 expression is physiologically relevant, it should cause coherent changes in DELLA accumulation with a daily rhythm. We focused our attention in two DELLA proteins, GA INSENSITIVE (GAI) and REPRESSOR OF ga1-3 (RGA), which are the most abundant DELLAs in young seedlings and shoots (20). To monitor the level of these proteins, we used lines that express the *RGA::GFP-RGA* (21) or *35S::TAP-GAI* (22) transgenes. Consistent with the clock regulation of *GID1* genes, both GFP-RGA and TAP-GAI protein levels oscillated in a diurnal manner, showing peak levels at the end of the light period (Fig. 1*B*). Strikingly, GFP-RGA oscillation was detected in the growing region of the hypocotyls (Fig. 1 *C–E* and *H–J*). Fluorescence from the fusion protein accumulated in nuclei of the uppermost part of hypocotyls at ZT9, whereas it was below the detection limit late in the night, at ZT18 (Fig. 1 *C–E*), coinciding with periods of minimum and maximum growth rates, respectively (4). The periodicity of RGA accumulation must largely be caused by the activity of GID1 receptors, as the RGA transcript did not show significant oscillation (Fig. S1*C*). Thus, given (*i*) the major role of RGA and GAI in controlling growth (23, 24) and (*ii*) that seedling growth under diurnal conditions is gated by the circadian clock (4), this result suggests that DELLA proteins are regulatory components for the control of the clock output, such as daily growth rhythm in young seedlings (4).

The oscillation of GFP-RGA levels was affected also in clock mutants. The waveform of GFP-RGA oscillation in *toc1-1* seedlings was slightly different from the WT, the amplitude was higher and the peak narrower because of an advance in the phase of the trough (Fig. 1 *G–J*), according to the phase advance observed in the expression of GA receptor genes (Fig. 1*F*). On the contrary, GFP-RGA protein levels were constant and low in the *lhy* mutant (Fig. 1 *L–O*), which correlates with the long hypocotyl phenotype observed in this mutant when grown in short days (3).

Circadian Clock Gates GA Signaling Activity. The observation that lower DELLA protein levels coincide with higher growth rates at the end of the night suggests that they participate in the core mechanism that controls rhythmic growth of hypocotyls. To test this hypothesis, we examined the impact upon growth of an alteration of the normal rhythm of GA signaling with two complementary approaches. First, we used a transgenic line that expresses a dominant version of GAI under the control of a heat-shock inducible promoter, *HS::gai-1D* (9). This line allowed us to block GA signaling by applying a 10-min heat shock at 33 °C at two different times of the day: ZT5, when the growth rate is low and DELLA levels high; and ZT17, which coincides with the beginning of the growing phase and with the trough of DELLA levels (Fig. 2*A*). The effectiveness of the treatments was con-

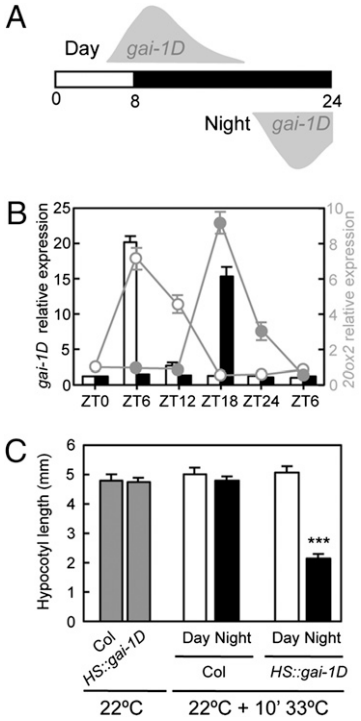

Fig. 2. Blocking GA signaling at night affects hypocotyl growth. Seedlings of the *HS::gai-1D* line were grown under short-day photocycles (8 h light/16 h dark) and received heat treatments of 33 °C for 10 min at ZT5 or ZT17 (*Materials and Methods*). In *A*, shaded areas mark the period of the day during which gai-1D accumulates. (*B*) Expression of *gai-1D* (bars) and its target gene *GA20ox2* (circles, scale on right) after heat treatments at ZT5 (white symbols) and ZT17 (dark symbols). (*C*) Hypocotyl length of Col-0 WT and *HS::gai-1D* seedlings that did not receive heat treatments (gray bars) or that received treatments at ZT5 (white bars) or ZT17 (black bars). The experiment was repeated three times with similar results. Data represent the mean ± SD ($n \geq 15$ seedlings), and asterisks indicate $P < 0.0001$.

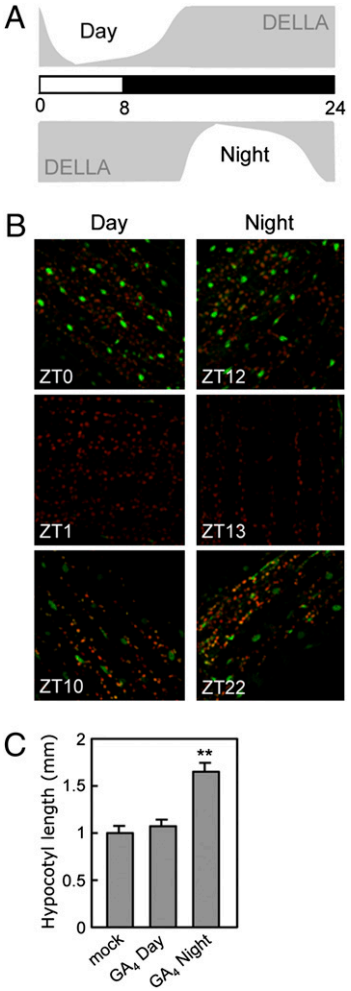

Fig. 3. GA application at night releases the growth restrain imposed by DELLAs. *RGA::GFP-RGA* seedlings grown under short-day photocycles (8 h light/16 h dark) in the presence of 0.2 μM PAC were treated with 1 μM GA_4 at ZT0 or ZT12 or untreated (*Materials and Methods*). (*A*) Scheme of DELLA accumulation after GA_4 treatments, deduced from the GFP-RGA fluorescence of seedlings grown under the same conditions (*B*). Confocal images taken at the time of GA_4 treatment (ZT0 and ZT12) and 1 h (ZT1 and ZT13) and 10 h later (ZT10 and ZT22) show that the maximum period with low DELLA levels spans less than 10 h. Images are representative of three independent biological repeats including eight to 10 seedlings per time point. (*C*) Hypocotyl length of WT (*Ler*) seedlings grown in the presence of 0.2 μM PAC that did not receive any additional treatment (mock) or that were treated with 1 μM GA_4 at ZT0 (day) or ZT12 (night). The WT seedlings contain the *RGA::GFP-RGA* transgene. Data represent mean ± SD ($n \geq 15$ seedlings). Asterisks indicate $P < 0.001$.

firmed by expression analysis of *gai-1D* and one of its known direct targets, *AtGA20ox2* (Fig. 2*B*). Interestingly, blocking GA activity at ZT5 during four consecutive days did not have any effect upon hypocotyl growth (Fig. 2*C*). On the contrary, the heat treatment had a strong inhibitory effect on the hypocotyl growth of *HS::gai-1D* seedlings when applied at ZT17 (Fig. 2*C*).

Second, we examined how transient application of GA_4 at two different times of the day would rescue the dwarfism caused by continuous incubation with paclobutrazol (PAC), a compound that causes accumulation of DELLA proteins (Fig. 3*A* and *B*). GA application provoked rapid degradation of DELLA proteins that lasted for the next 10 h: between ZT1 and ZT10 when the GA treatment was applied at dawn, and between ZT13 and ZT22 when applied at ZT12 (Fig. 3*B*). Importantly, GA treatment applied at ZT12 during two consecutive days (fourth and fifth) significantly alleviated the growth-repressing effects of PAC, whereas it had no effect when applied at dawn (Fig. 3*C*). In summary, these results confirm that there is a DELLA-sensitive period that overlaps the growing phase of the night, and that under short-day conditions the circadian clock might allow growth by preventing accumulation of DELLA proteins during that particular period.

Oscillation of GA Signaling Refines Rhythmic Growth. If the oscillation of GA signaling constitutes part of the mechanism that ensures rhythmic growth, a prediction of this model is that GA signaling mutants should display not only a defect in the final size of the hypocotyl (12), but also an altered rhythmic growth pattern. In agreement with this, transgenic plants that expressed *GID1a* under the control of the *35S* promoter showed an expanded growth phase that started at the same time as in the WT and extended well into daytime, almost spanning the whole light period (Fig. 4*A*). In agreement with this, plants overexpressing *GID1a*, *GID1b*, or *GID1c* displayed longer hypocotyls compared with the WT ($P < 0.05$, Student *t* test; Fig. S3*A*), and seedlings of *gid1a-1*, *gid1b-1*, and *gid1c-1* loss-of-function mutants, and of the different double mutant combinations (17), had shorter hypocotyls than WT ($P < 0.05$, Student *t* test; Fig. S3*B*). Taken together, these results indicate that *GID1* expression is limiting for promotion of hypocotyl elongation under diurnal conditions and that oscillation of *GID1* is necessary for the establishment of proper patterns of rhythmic growth.

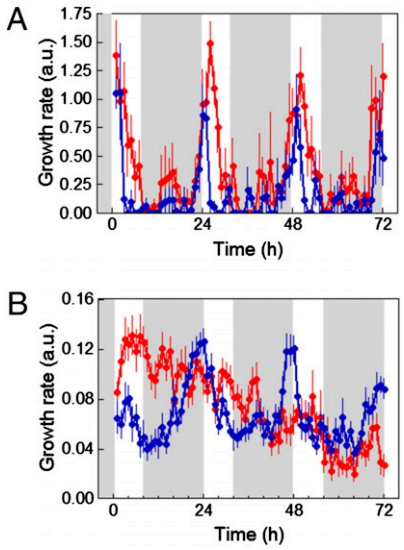

Fig. 4. GA activity regulates diurnal rhythms of hypocotyl elongation. Col 0 and *35S::GID1a* seedlings (*A*) and Ler and quintuple *della* mutant seedlings (*B*) were grown under short-day photocycles (8 h light/16 h dark) for 3 d before they were imaged under the same conditions for three additional days. Blue and red symbols and lines denote the WT and mutant/transgenic seedlings, respectively. Seedlings' growth rates were measured as described in *Materials and Methods*. White and gray areas represent day and night, respectively. Data represent mean ± SD ($n \geq 10$ seedlings). a.u., arbitrary units.

Nonetheless, seedlings overexpressing *GID1a* still exhibited robust rhythmic growth. Given that this behavior cannot be attributed to circadian regulation of GID1 protein stability (Fig. S4), it could be taken as an indication that GA levels oscillate in a diurnal or in a clock-controlled manner. In fact, expression of several genes encoding GA metabolic enzymes oscillate diurnally in *Arabidopsis* (25, 26), in a manner that would likely result in lower GA levels at dusk; and diurnal changes in GA levels have been described in sorghum (27).

More clear evidence for an integral role of GA signaling in the establishment of rhythmic growth was provided by the quintuple *della* KO mutant (22). Growth of this mutant was completely arrhythmic under short days, showing a relatively high growth rate during the first day examined that progressively decreased over the next few days (Fig. 4*B*). This arrhythmic phenotype is not likely caused by a dysfunction of the core clock mechanism, as the expression of the clock genes *TOC1* and *CCA1* was not significantly affected in the mutant (Fig. S5). DELLAs have been proposed to regulate cell expansion through the inhibition of PIF's activity (22, 28). The observation that *pif4 pif5* double mutants are impaired in rhythmic growth (4) suggests that the DELLA-PIF interaction might thus constitute the main output pathway that controls rhythmic growth. However, given that DELLAs also exert part of their action through the HY5 transcription factor (9) and *hy5* mutants display arrhythmic growth (4), it is also possible that other components different from PIF might also be part of this regulatory module.

DELLA Proteins Mediate Daily Rhythms of Gene Expression. GA signaling participates in the regulation of multiple developmental and physiological processes other than cell expansion (29). It is therefore possible that the role of DELLA proteins as regulatory components of the output of the circadian clock extends beyond the control of rhythmic growth and affects other processes. To test this hypothesis, we performed a genome-wide search for genes that would oscillate in a circadian manner in WT seedlings, and whose oscillation would be affected in a *della* KO mutant. For this purpose, we examined global gene expression in short-day–grown WT and *della* mutant seedlings at ZT9 (high DELLA levels) and ZT21 (low DELLA levels). In this search, we found 5,087 genes whose expression varied between both time points in the WT. We found that this list was significantly enriched (66%; $P < 2.2 \times 10^{-16}$ by Fisher exact test) for genes differentially expressed across time points in a previously published full short-day data set (2, 13), indicating that our approximation to identify genes that oscillated with a specific diurnal phase was acceptable. To ask what role DELLA genes play in diurnal regulation of gene expression, we looked for genes that were differentially expressed when *della* mutants were compared with WT. We found 58 genes differentially expressed between *della* and WT; 37 of these were only found at ZT9 and four were found at ZT21, consistent with DELLAs being most important at ZT9 (Fig. 5*A* and Dataset S1). Furthermore, the 37 genes affected by the *della* KO at ZT9 were significantly enriched for diurnally regulated genes (70%; $P = 0.001$ by Fisher exact test), whereas the remaining differentially expressed genes were not enriched.

Importantly, Gene Ontology analysis showed that, among the genes whose oscillation was altered in the *della* mutant, there was an enrichment in functional categories related to the response to stress and environmental signals, as well as in genes encoding proteins located in the cell wall and the endoplasmic reticulum (Fig. 5*B*). Although part of the genes represented in the "cell wall" category could include those with a function in growth and cell expansion, the enrichment of other growth-unrelated categories suggests that DELLAs mediate the regulation of a larger array of circadian clock-controlled processes. These results at-

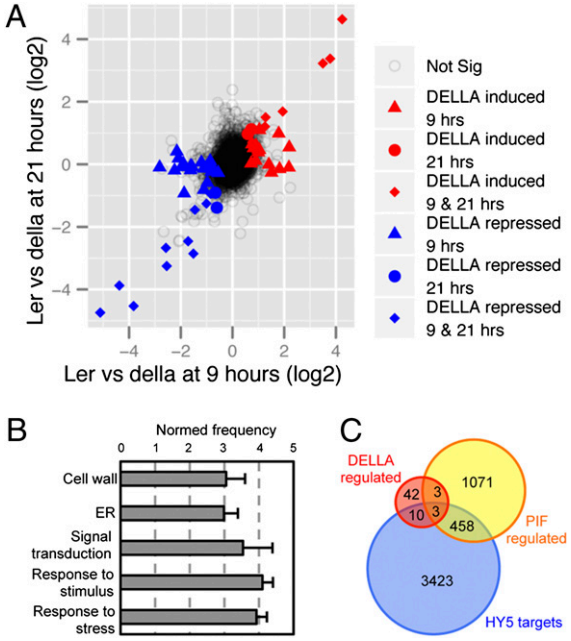

Fig. 5. DELLAs mediate circadian regulation of transcription. (*A*) Scatter plot of genes differentially regulated at ZT21 versus ZT9 in WT Ler and *della* mutants. Genes showing statistically significant (FDR < 0.1) differential expression between *Ler* and *della* are displayed for each time point in blue and red. (*B*) Enrichment of gene ontology categories among genes regulated by DELLAs at ZT9 ($P < 0.0001$ in all cases). (*C*) Venn diagram showing overlap between genes regulated by DELLAs at ZT9; genes directly bound by HY5, as detected by ChIP-chip experiments in light-grown seedlings (30); and genes regulated by PIF transcription factors, as genes differentially expressed in the quadruple *pif1 pif3 pif4 pif5* mutant in darkness and light, with respect to WT (31).

tribute a more general role to DELLA proteins in the modulation of the output of the clock. Moreover, meta-analysis of the DELLA targets at ZT9 (Fig. 5*C*) indicated that only 45% of these genes were either direct targets for HY5 (30) or genes also regulated by the PIF transcription factors (31, 32). This implies that DELLA proteins control gene expression through the interaction with additional transcription factors, which is in agreement with the observation that DELLA proteins can interact with several members of the bHLH family of transcription factors other than PIFs (33, 34).

Concluding Remarks. The enormous plasticity of plant growth and development is based on a web of interacting signaling pathways, which provides the plant with multiple entry points to adjust their physiology in response to frequent, unpredicted environmental changes (35). The circadian system, on the contrary, provides the plant with the ability to anticipate predictable daily and seasonal environmental changes (36) and buffers plant responses against casual environmental variability (37). The circadian clock therefore provides stability to plants' life. Then, can responses regulated by the circadian clock be plastic? We suggest that the regulation of GA activity by the circadian clock might provide such ability, acting as a link between two properties critical for plant growth and development, robustness, and plasticity (38). Thus, the concurrency of clock and GA regulation of certain processes guarantees a precise and robust response to unpredicted, transitory, and above-noise changes in the environment that have an impact on the GA pathway, such as nutrient availability (39), salt stress (18), ambient temperature (40), or flooding (41). The observation that the functioning of the circadian clock does not seem to be affected significantly by GA (ref. 42 and the present study) supports the suggested role of GA as a regulatory output module that fine-tunes clock-regulated gene expression in response to environmental signals.

Materials and Methods

Plant Material. *Arabidopsis thaliana* accessions Col-0 and L*er* were used as WT. Seeds of gid1a-1, gid1b-1, gid1c-1, gid1a-1 gid1b-1, gid1b-1 gid1c-1, gid1a-1 gid1c-1, toc1-1, lhy, rga-t2 gai-t6 rgl1-1 rgl2-1, rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1, RGA::GFP-RGA, *35S*::TAP-GAI, and HS::gai-1D have been previously described (9, 15, 17, 18, 21, 22, 43). RGA::GFP-RGA toc1-1 and RGA::GFP-RGA lhy lines were obtained by genetic crosses and isolated from an F_3 population.

Plasmid Constructs and Transgenic Plants. The preparation of transgenic lines expressing *35S::GID1a-YFP-HA*, *35S::GID1b-YFP-HA*, or *35S::GID1c-YFP-HA* was as follows. Coding sequences of *GID1a-1c*, excluding the stop codon, were PCR-amplified with Pwo polymerase (Roche) from cDNA obtained from 7-d-old, light-grown WT Col-0 seedlings. Oligonucleotides used as primers for PCR (Dataset S2) included the *att*B sites needed for Gateway-mediated cloning and were designed to allow expression of a C-terminal fusion. PCR products were first cloned into vector *pDONR-221* (Invitrogen) by BP recombination, and then transferred to the binary vector *pEarleyGate-101* (44) by LR recombination to create a C-terminal fusion with YFP and HA-tag. The final constructs were transferred to WT Col-0 plants by *Agrobacterium*-mediated transformation. Primary transformants were selected in MS plates containing 50 mM glufosinate ammonium (Fluka). Transgenic lines with a 3:1 (resistant: sensitive) segregation ratio were selected, and several homozygous lines were identified in the T_3 generation for each construction.

Seedling Growth Assays. All seeds were surface-sterilized with 70% (vol/vol) ethanol and 0.01% (vol/vol) Triton X-100 for 5 min, followed by 96% (vol/vol) ethanol for 5 min. Seeds were sown on plates of 0.5 MS medium (Duchefa) and 0.8% (wt/vol) agar without sucrose, and stratified at 4 °C in darkness for 5 d. Germination was induced by placing the plates under white fluorescent light (190–200 μmol m^{-2} s^{-1}) at 22 °C for 8 h. Seedlings were grown at 22 °C under short-day photocycles, 8 h light (70–200 μmol $m^{-2}s^{-1}$, depending on the experiment)/16 h dark in an E-30B growth cabinet (Percival).

For heat-shock experiments, WT Col-0 and *HS::gai-1D* (9) seedlings grown in the same plate under short days (70 μmol m^{-2} s^{-1}) received a heat treatment (10 min at 33 °C in darkness) at either ZT5 or ZT17. Control seedlings of both genotypes were kept at 22 °C. Heat treatments were applied at days 3, 4, 5, and 6. Hypocotyl length was measured on day 7.

For GA sensitivity assays, *RGA::GFP-RGA* seeds were sown on sterile filter papers placed on 0.5 MS, 0.8% (wt/vol) agar plates without sucrose, stratified, and induced to germinate as described earlier. After induction of germination, filter papers harboring seeds were transferred to treatment plates containing 0.2 μM PAC (Duchefa) and grown under short days (190–200 μmol m^{-2} s^{-1}) at 22 °C for 3 d. Filter papers containing 4-d-old seedlings were transferred at either ZT0 or ZT12 for 1 h to Petri dishes with 5 mL of 0.5 MS liquid media containing 0.1 μM GA_4 (Sigma) plus 0.2 μM PAC, or just 0.2 μM PAC. Filter papers containing seedlings were then rinsed three times for 20 min in Petri dishes containing 0.5 MS with PAC 0.2 μM. After washes, seedlings were transferred into a new sterile filter paper, placed on a fresh 0.2-μM PAC treatment plate, and returned to short-day conditions. GA treatments were given during two consecutive days (fourth and fifth), and hypocotyl length was measured on day 6. Handling of seedlings during the dark period was performed under a safe green light.

To measure hypocotyl length, seedlings were placed on an acetate sheet and scanned at a resolution of 600 dpi, and the length was measured with ImageJ software.

The time-lapse photography and image analysis to determine hypocotyl growth rate was performed as previously described (4), except that the growth medium contained 0.5 MS and 1% sucrose.

RNA Extraction and Gene Expression Analysis by Quantitative RT-PCR. Total RNA was isolated from whole seedlings grown as described earlier (190–200 μmol m^{-2} s^{-1}) by using the E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek) according to the manufacturer´s instructions. cDNA synthesis and quantitative PCR conditions were as described (9). Primers used are listed in Table S2.

Microarray Analysis. WT L*er* and *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* pentuple *della* mutant seedlings were grown under short days (190–200 $\mu molm^{-2}s^{-1}$) at 22 °C, and seedlings were sampled at ZT9 and ZT12 of day 5. Total RNA from whole seedlings was extracted with RNeasy Plant Mini kit (Qiagen). RNA labeling and hybridization to Affymetrix *ATH1* arrays were performed by the Nottingham *Arabidopsis* Stock Centre. Analysis was performed in R (45) and Bioconductor (46). Microarrays were normalized with the RMA procedure as implemented in the affy package (47), and differential expression was determined using limma (48, 49) with a false discovery rate lower than 0.05. To determine genes whose expression varies in diurnal short-day conditions, the previously published data set (13) was downloaded from Array Express (http://www.ebi.ac.uk/arrayexpress/; accession E-MEXP-1304), Robust Multichip Average-normalized, and analyzed in limma by using a one-way ANOVA model with time as the grouping variable. Samples from each of the 2 d of collection were used as replicates (so, in total, there were two replicates for each of six time points). Gene annotations were based on the TAIR9 version of the *Arabidopsis* Web site (http://arabidopsis.org). Gene Ontology analysis was performed with the Classification SuperViewer tool at the Bio-Array Resource of the University of Toronto.

Protein Extraction and Western Blot Analysis. Protein extraction and Western blot analysis from whole 5-d-old seedlings grown under short days (190–200 μmol m^{-2} s^{-1}) were performed as described. The GFP, TAP, and HA fusion proteins were detected using anti-GFP (JL8; Clontech), anti–c-myc (9E10; Roche), and anti-HA (3F10; Roche) antibodies, respectively. Antibodies against DET3 were used to check protein loading (50). Signal from bound antibodies was revealed using ECL Advance Western Blotting detection Kit (GE Healthcare) and visualized and quantified by using the Luminescence Image Analyzer LAS-3000 (Fujifilm) and Image Gauge (version 4.0; Fujifilm), respectively.

Confocal Imaging. Fluorescence from the GFP-RGA fusion protein was detected by using a confocal microscope (TCS SL; Leica) as previously described (40).

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Supporting Information

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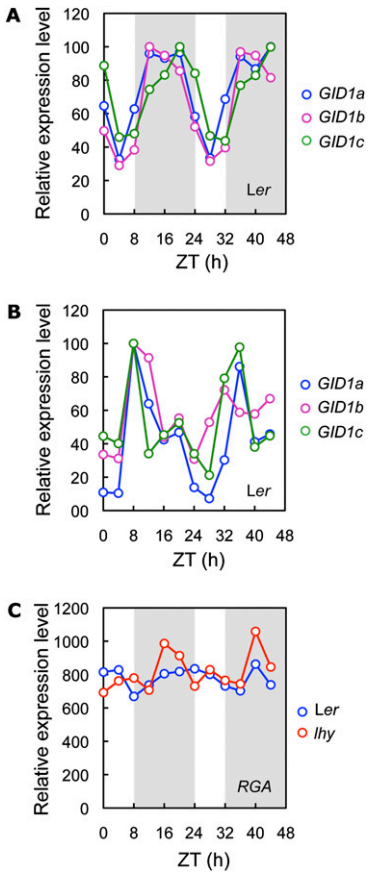

Fig. S1. The circadian clock controls diurnal oscillation of *GID1* genes in *Arabidopsis*. (*A*) Transcript levels of *GID1* genes in short-day photocycles (8 h light/16 h dark). (*B*) Transcript levels of *GID1* genes in continuous light after entrainment in short-day photocycles. (*C*) Expression of *RGA* in WT and *lhy* mutant seedlings in short-day photocycles. Data are taken from DIURNAL (http://diurnal.cgrb.oregonstate.edu/) and are normalized to the average value to facilitate comparison.

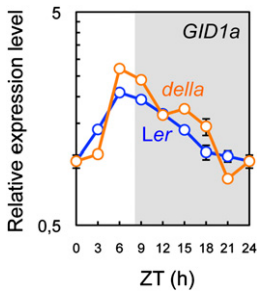

Fig. S2. Expression of *GID1a* in 5-d-old L*er* WT and in quadruple *della* mutant seedlings grown under short-day photocycles (8 h light/16 h dark). Values are expressed relative to *PP2a* expression. Data represent mean ± SD of three technical replicates. Experiments were repeated twice with similar results. White and gray areas represent day and night, respectively.

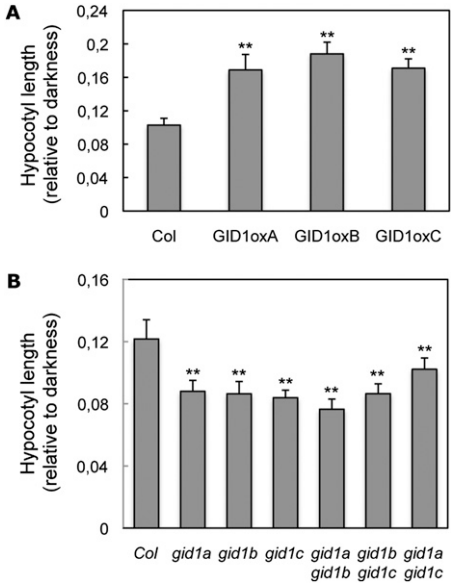

Fig. S3. GID1 activity controls hypocotyl elongation under short-day conditions. (*A*) Normalized hypocotyl length of Col-0 WT and *35S::GID1a*, *35S::GID1b* and *35S::GID1c* seedlings grown for 7 d in constant darkness and in short-day photocycles (8 h light/16 h dark). (*B*) Normalized hypocotyl length of Col-0 WT and *gid1* mutant seedlings grown for 7 d in constant darkness and in short-day photocycles (8 h light/16 h dark). Data are mean ± SD ($n \geq 15$ seedlings, $^{**}P < 0.001$). Hypocotyl length under diurnal conditions was normalized to that in etiolated seedlings. Experiments were repeated twice with similar results; results from one representative experiment are shown.

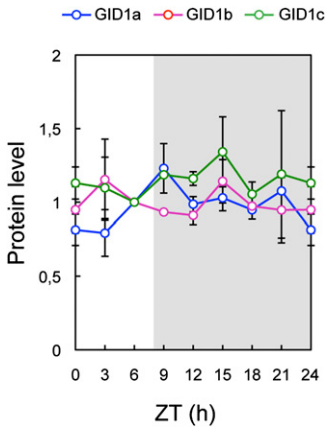

Fig. S4. GID1s accumulate constitutively in the overexpressing lines. *35S::GID1a*, *35S::GID1b*, and *35S::GID1c* seedlings were grown for 5 d in short-day photocycles (8 h light/16 h dark). GID1-GFP protein levels were determined by Western blot with commercial antibodies against GFP. GID1 levels were normalized against levels of DET3, which was used as loading control. Data are the mean of two independent experiments. White and gray areas represent day and night, respectively.

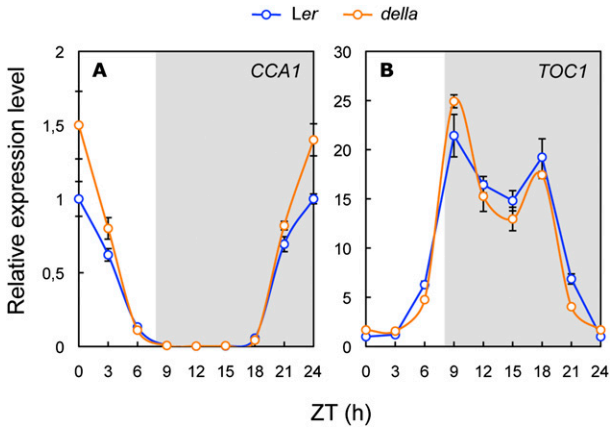

Fig. S5. The core clock mechanism is not affected in the pentuple *della* mutant. WT L*er* and pentuple *della* seedlings were grown under short-day photocycles (8 h light/16 h dark) for 5 d. Expression of *CCA1* (*A*) and *TOC1* (*B*) clock genes is shown. Values are expressed relative to *PP2a*. Data represent mean and SD of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown. White and gray areas represent day and night, respectively.

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)