**Title:**

**GIGANTEA links endogenous timing to light and hormone signaling pathways**

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**Abstract**

Integration of environmental signals with endogenous biological processes is essential for organisms to thrive in their natural environment. Being entrained by light and temperature oscillations, the circadian clock incorporates ambient information to coordinate multiple physiological processes and phase them to the optimal time of the day and year. Here we show how the core clock component GIGANTEA (GI) integrates endogenous timing with light and hormone signaling pathways through the modulation of the PHYTOCHROME-INTERACTING FACTORs (PIFs) and the DELLA proteins, which are key regulators of these pathways. Gating of the activity of these transcriptional regulators by GI directly affects output rhythms such as photoperiodic growth. In addition, we uncover a role for the PIFs in the circadian clock and show how their regulation by GI is required to adequately set the pace of the clock.

**Introduction**

Proper decoding of environmental signals and integration of these cues within cellular networks is essential for organisms to succeed in their natural environment. The rhythmic and periodic nature of relevant external conditions, such as light/dark cycles and ambient temperature oscillations, has fostered the evolution of endogenous timekeeping molecular oscillators that enable organisms to predict and anticipate to these cyclic changes (Millar, 2016). Being entrained by ambient light and temperature signals (Nohales & Kay, 2016), these circadian clocks coordinate key molecular and physiological processes to occur at the optimal time of the day and year, hence allowing for efficient use and distribution of resources (Millar, 2016).

In plants, adequate integration of environmental cues and precise phasing of biological processes become especially valuable features, since their sessile nature impedes them to escape disadvantageous conditions. The influence of the circadian clock on plant development is pervasive and multiple processes, including growth, biotic and abiotic stress responses, and developmental transitions, are coordinated by the clock in conjunction with other signaling pathways (Greenham & McClung 2015, Sanchez & Kay 2016). Photoperiodic growth exemplifies a case of a key developmental process that is regulated by the concerted action of the endogenous clock, light perception and hormone signaling (de Montaigu et al. 2010, Farre 2012).

GI (~~Redei, 1962~~; Park et al. 1999, Fowler et al. 1999) is a conserved plant-specific core clock protein expressed in the evening. It is essential for accurate timekeeping (Locke et al. 2005, Mizoguchi et al. 2005, Gould et al. 2006, Martin-Tryon et al. 2007, Kim et al. 2007), and is involved in blue and red light input to the clock (Park et al. 1999, Martin-Tryon et al. 2007) and temperature compensation (Gould et al. 2006). Besides its role in the central oscillator, GI modulates myriad clock output pathways, that range from abiotic stress responses (Cao et al. 2005, Kim et al. 2013) to flowering time determination (Suarez-Lopez et al. 2001, Sawa et al. 2007), and light signaling and hypocotyl elongation (Huq et al. 2000, Oliverio et al. 2007, Martin-Tryon et al. 2007). Despite its pivotal implication in clock function and plant development, knowledge on how GI is able to influence such a wide array of cellular networks remains largely unexplored.

Here we show how GI modulates light and hormone signaling pathways by gating the activity of the PIF and DELLA proteins, two of their key regulators (Castillon et al. 2007, Zhang et al. 2013, Sun 2011, Hauvermale et al. 2012). At the physiological level, we also provide evidence on how this regulation influences output rhythms such as photoperiodic growth. Since these proteins function as hubs in the regulation of plant growth and development (Leivar & Quail 2011, Xu et al. 2014, Daviere & Achard 2016), modulation of their activity by GI provides a means by which the circadian clock gates the sensitivity to external and internal signals to ultimately shape plant growth and development. Moreover, we uncover a role for the PIF proteins in the circadian system and show that their regulation by GI is required for proper clock progression, providing a molecular framework for light input into the circadian clock.

**Results and Discussion**

**GI shares targets with both clock and light signaling pathways**

Not harboring well characterized functional domains, the molecular mechanisms by which GI is able to exert its wide regulatory function remain unclear. GI has been reported to interact with multiple proteins that function in diverse pathways (Mishra & Panigrahi 2015), suggesting a possible role as a scaffold protein. In the context of transcriptional regulation, GI has been shown to influence transcription of flowering time genes through interaction with and modulation of their transcriptional regulators, as well as by occupancy of promoter regions (Sawa et al. 2007, Sawa & Kay 2011). To further investigate the molecular mechanisms through which GI impinges on gene regulatory networks for the coordination of the circadian system and its output, we initiated our study by performing a reanalysis of published genome-wide gene expression datasets (Kim et al. 2012, Oh et al. 2012, Kamioka et al. 2016). A search for over-represented *cis* elements at the promoter regions of differentially expressed genes (DEGs) in *gi-2* mutant seedlings (Kim et al. 2012) revealed the G-box [CACGTG] and the evening element (EE) [(A)AAATATCT] as the two most highly enriched motifs (Suppl. Fig. 1a).

The G-box motif is found in the promoters of many light regulated genes and is known to be bound by different transcription factors including the PIFs (Jakoby et al. 2002, Martinez-Garcia at al. 2000, Huq & Quail 2002, Hornitschek et al. 2009), whereas the EE is bound by the morning core clock repressors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Alabadi et al., 2001) and is enriched in the promoters of genes with peak expression in the evening (Harmer et al. 2000, Covington et al. 2008, Harmer 2009). We subsequently analyzed the overlap between genes regulated by GI (Kim et al. 2012) and those regulated by either PIFs (Oh et al. 2012) or CCA1 and LHY (Kamioka et al. 2016). This analysis rendered two subsets of shared genes with distinct characteristics (Fig. 1a, Suppl. Fig. 1b). The subset of genes shared by GI and PIFs is enriched in Gene Ontology (GO) terms related to photosynthesis, response to hormone, and light signaling pathways (Suppl. Fig 1c), and is enriched in peak expression phases around dawn (Zeitgeber time 23 (ZT23) to ZT1), especially under short day (SD) conditions (Fig. 1b, Suppl. Fig. 1d). On the contrary, genes shared by GI and CCA1 and LHY are involved in pathways related to circadian rhythms, temperature acclimation, response to diverse abiotic stimuli, and response to hormones (Suppl. Fig. 1c). In this case, analysis of time-of-day expression also revealed that these genes tend to be expressed in the evening, mostly at ZT10 to ZT14 (Suppl. Fig. 1e).

These results support the notion that GI plays dual roles in the regulation of light signaling and the circadian system, as earlier proposed (Mizoguchi et al. 2005, Martin-Tryon et al. 2007, Oliverio et al. 2007). Genes shared by GI and CCA1/LHY are clock components and clock outputs whose expression is likely driven by the EE, in accordance with the role of GI in the central oscillator and in the regulation of *CCA* and *LHY* amplitude (Park et al. 1999, Fowler et al. 1999, Mizoguchi et al. 2002). Conversely, GI and PIFs seem to intersect at a subset of genes for the regulation of photosynthesis, light signaling, and growth promotion at the end of the night.

**GI interacts with PIFs and their negative regulators the DELLA proteins**

GI acts as a positive regulator of light signal transduction and photomorphogenesis (Huq et al. 2000, Martin-Tryon et al. 2007), and *gi* mutant plants display a long hypocotyl phenotype under different conditions (Suppl. Fig. 2). Given the ability of GI to interact and modulate the activity of diverse proteins, we hypothesized that interaction with PIF proteins may reside at the core of the mechanism by which GI regulates light signaling transcriptional networks.

Leveraging an arrayed *Arabidopsis* transcription factor library (Pruneda-Paz et al. 2014), we performed a yeast two-hybrid screen using GI as a bait. We found that GI is able to strongly interact with PIF3, and in a weaker manner with other PIFs such as PIF1, PIF4 and PIF5, in this heterologous system (Suppl. Fig 3 a, c). Interestingly, we also detected interaction of GI with the DELLA proteins REPRESSOR OF GA1-3 (RGA), GIBBERELLIC ACID INSENSITIVE (GAI) and RGA-LIKE PROTEIN 3 (RGL3), which are negative regulators of gibberellin (GA) signaling and known repressors of the PIFs (de Lucas et al. 2008, Feng et al. 2008) (Suppl. Fig 3 b, c). These results raised the possibility that the mechanism underpinning GI promotion of light signaling and inhibition of hypocotyl elongation involved modulation of PIF proteins directly and/or indirectly through the DELLA proteins.

Through *in vitro* pull-down assays using full-length and deleted protein fragments, we verified the observed interactions (Fig. 1c, d), and mapped the interaction domains for PIF3 and RGA (Suppl. Fig. 4a, b). These deletion studies showed that GI interacts with PIF3 through its bHLH DNA-binding motif (Suppl. Fig. 4a), suggesting that the interaction could hinder PIF3 binding to the chromatin. In the case of RGA, the experiments revealed that all partial fragments except for the DELLA domain where able to interact with GI (Suppl. Fig. 4b). Attending to the recently published crystal structure of the SCARECROW-LIKE PROTEIN 7 (SCL7) GRAS domain (Li et al. 2016), it is possible that the deleted RGA portions interacting with GI contain parts of the region mediating protein-protein interactions at its GRAS domain. We further confirmed the GI-PIF interaction *in vivo* by performing co-immunoprecipitation (co-IP) studies in transgenic *Arabidopsis thaliana* seedlings expressing tagged protein versions (GI-YPET-Flag;PIF3-ECFP-HA and GI-YPET-Flag;PIF5-HA) (Fig. 1e). The interaction between Flag-GI and HA-RGA was also confirmed *in planta* by transient expression and co-IP assays in *Nicotiana benthamiana* leaves (Fig. 1f). Furthermore, transient co-expression of GI-YPET-Flag, PIF3/5-ECFP-HA and RGA-mCherry-cMyc revealed that they co-localize in defined nuclear bodies (Fig. 1g, Suppl. Fig. 5).

**GI stabilizes DELLAs and is involved in the circadian gating of GA signaling**

Because several pathways in which GI is involved comprise protein stability (Sawa et al. 2007, Kim et al. 2007), and both GI and RGA coincide and peak towards the end of the light period (David et al. 2006, Arana et al. 2011), we wondered if GI interaction with RGA could be contributing to RGA balance. Stability analyses in transient expression in *N. benthamiana* leaves revealed that RGA-GFP protein levels are stabilized in the presence of GI, similarly to the effect caused by a treatment with the proteasome inhibitor MG-132 (Supp. Fig. 6a). To further investigate how GI contributes to shape the diurnal pattern of RGA accumulation, we crossed an *Arabidopsis* transgenic line expressing GFP-RGA driven by its endogenous promoter (Silverstone et al. 2001) into the *gi-2* mutant background and a GI overexpression line (GIox) (David et al. 2006). Western blot analysis of the protein levels across the diurnal cycle in SD conditions confirmed that GFP-RGA accumulation is enhanced by GI overexpression, remaining high even during the night phase, while it is compromised and low across the entire day in its absence (Fig. 2a, Suppl. Fig. 6b). Accordingly, loss of *RGA* and *GAI* was observed to alleviate the short hypocotyl phenotype of GIox lines (Fig. 2b), demonstrating that higher DELLA activity contributes to the growth phenotype in the GI overexpression lines.

At the mechanistic level, we reasoned that GI binding to RGA in the evening could hinder access of the GA receptor GA INSENSITIVE DWARF 1 (GID1) to it, hence interfering with its degradation. Upon GA sensing, the GID1A receptor undergoes a conformational change that increases the affinity for its ligands the DELLA proteins and promotes binding to them through the DELLA domain (Murase et al. 2008, Sun 2011). *In vitro* pull-down studies of the GID1A-RGA binding in the absence and presence of GI confirmed that GI negatively affects this interaction (Suppl. Fig. 7a). Moreover, increasing quantities of GI progressively decreased the amount of RGA co-immunoprecipitated with GID1A (Fig. 2c). In addition, GFP-RGA degradation time-course experiments in *Arabidopsis* seedlings treated with the GA GA3 showed that GFP-RGA degrades faster in *gi-2* mutants compared to wildtype plants (Fig. 2d), and this difference is abolished in the presence of MG-132 (Suppl. Fig. 7b). These observations support the role of GI as a stabilizing partner of RGA that gates its sensitivity to degradation through the GID1 pathway. In accordance with this notion, accumulation of RG52, a mutated version of RGA lacking the DELLA domain, was not affected by the presence or absence of GI in *N. benthamiana* leaves (Suppl. Fig. 7c), likely because this variant is insensitive to degradation by GID1 and already stable. Furthermore, while plants expressing wildtype (degradation-sensitive) RGA fused to GFP display longer hypocotyls in *gi-2* plants (and lower RGA-GFP content, as described above), no difference was observed between wildtype and *gi* mutant plants expressing a dominant negative DELLA protein (Suppl. Fig. 7d).

Given that DELLAs are negative regulators of GA signaling (Sun 2011, Hauvermale et al. 2012), imbalance in RGA levels in *gi-2* mutants is expected to affect signaling of this hormone. Consistently, a dose response curve in the presence of GA3 and the inhibitor of GA synthesis paclobutrazol (PAC) showed that *gi-2* mutants have indeed altered GA signaling, being hypersensitive to GA3 and hyposensitive to PAC (Suppl. Fig. 8a). Because both *gi-2* and GIox plants do not show major perturbations in the expression of GA metabolism and signaling components (Suppl. Fig. 8b), the GA signaling phenotype observed in *gi* mutants is likely due to the reduced DELLA protein accumulation and activity. In light of these results, we wondered if GI and, by extension, GI-mediated RGA stabilization may be involved in the circadian gating of GA sensitivity (Arana et al. 2011). To test this, we treated wildtype and *gi-2* plants with GA4 at different times of the day and measured the effect of this treatment on hypocotyl elongation compared to mock treatments. As anticipated, treatment at ZT12 had the strongest effect in wildtype plants (Arana et al. 2011). In contrast, *gi-2* mutants were responsive to the treatment across the entire night period (Fig. 2e) regardless of the time of application, suggesting that GI plays a role in the gating of this response. Considering the DELLA-stabilizing effect of GI, these results imply that GI gates GA sensitivity through the modulation of DELLA susceptibility to degradation. Further supporting the DELLA dependence of the observed phenotypes, induction of expression of a dominant negative version of GAI during the night strongly suppressed the long hypocotyl phenotype of *gi-2* mutants (Suppl. Fig. 8c). Hence, we conclude that, at the end of the day, GI interaction with RGA and probably other DELLA proteins stabilizes them and keeps responsiveness to GAs low. As the dark period progresses, GI degradation releases RGA and allows access of the GID1 receptor to it, which promotes its degradation and triggers the GA signaling cascade.

**GI represses PIF activity and hinders their access to target chromatin**

It has been previously reported that interaction of the DELLA repressors with PIF proteins interferes with their DNA-binding activity through a sequestration mechanism (de Lucas et al. 2008; Feng et al. 2008). Given the effect of GI on RGA stability, we hypothesized that GI could function in the same pathway as the DELLA proteins, binding PIF transcription factors and preventing their access to target genes. Gene expression analyses of PIF targets revealed that these are significantly altered in *gi-2* and GIox plants (Fig. 3a, Suppl. Fig. 9a), even though GI does not seem to greatly contribute to PIF mRNA expression or protein stability (Suppl. Fig. 9b, c). These observations further indicated that GI may regulate PIF target gene expression through modulation of PIF activity and/or accessibility to target promoters. To test this hypothesis, we performed transient transcriptional activation assays and measured activation of the *pPIL1*::LUC reporter as a proxy for PIF activity (Nieto et al. 2015). Because PIF3 activity could not be assessed under our experimental conditions (Suppl. Fig. 10a), we monitored PIF1, PIF4, and PIF5 activity. In all cases, PIF expression resulted in activation of the reporter as expected (Fig. 3b, Suppl. Fig. 10b). Co-expression of GI together with the PIF effectors, however, led to a drastic reduction in *pPIL1* promoter activation (Fig. 3b, Suppl. Fig. 10b). Strikingly, GI showed a much more severe effect on *pPIL1* activation than the one observed for RGA (Fig. 3b, lower panel), even though both proteins were expressed at similar levels (Suppl. Fig. 10c). In addition, co-expression of GI and RGA resulted in much stronger inhibition of PIF5 activity compared to RGA alone (Fig. 3b, lower panel).

To assess the effect of GI on PIF binding to target promoters *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays in *Arabidopsis* transgenic lines expressing PIF3-ECFP-HA in both wildtype and *gi-2* backgrounds. As shown in Fig. 3c, we observed a greater enrichment of the G-box containing regions in the promoters of the well-characterized PIF target genes *PHYTOCHROME INTERACTING FACTOR 3-LIKE 1* (*PIL1*) and *LONG HYPOCOTYL IN FAR-RED, REDUCED PHYTOCHROME SIGNALING 1* (*HFR1*) in the immunoprecipitated fractions in the absence of GI compared to wildtype plants, despite both lines showing similar levels of PIF3-ECFP-HA protein (Suppl. Fig. 10d). These results further support the role of GI in modulating PIF accessibility to target promoters. Since previous reports have shown that GI is able to associate with the chromatin (Sawa et al. 2007, Sawa & Kay 2011), and given the strong effects of GI on *pPIL1* activation in the transient activation assays, we decided to investigate whether GI may additionally sterically compete with PIF proteins for common chromatin target sites. We therefore analyzed GI occupancy of the promoter regions known to be bound by PIFs in *Arabidopsis* transgenic lines expressing GI fused to an YPET-Flag tag. We observed that GI occupies PIF target regions at dusk (ZT8) (Fig. 3d), when PIFs are not yet found to be bound to their targets (Soy et al. 2016). Later in the night (ZT16), these sites are released from GI binding (Fig. 3d), and hence made accessible for PIFs.

Altogether, our results suggest a role for GI in gating the occupancy of PIF proteins on their genomic target sites during the light-dark transition not only through direct interaction, but also through competition for *cis* elements. This implies that differential activity and binding of PIF proteins to their target promoters underlies the increased expression levels of *PIL1*, *HFR1*, and other PIF target genes observed in *gi-2* mutant plants (Fig. 3a, Suppl. Fig. 9a). In line with this, loss of *PIF3*, *PIF4*, and *PIF5* drastically reduced the expression of *PIL1* and *HFR1* in *gi-2* seedlings grown under SD conditions (Fig. 3e, f). This molecular phenotype also translates in a reduction of hypocotyl elongation at the phenotypical level (Fig. 3g). Hence we conclude that GI restricts PIF regulated processes such as growth by preventing PIF access to the chromatin during the early night in SDs; successive degradation of GI in the dark and subsequent release of those target regions allow PIFs to bind and, in the case of growth promoting genes, activate them at the end of the night.

**GI modulation of light signaling affects output and circadian rhythms**

The observed molecular and physiological interactions between GI and the PIF-DELLA module evidence how the circadian clock and light and hormone signaling pathways intersect for the regulation of a physiological output such as growth. By measuring hypocotyl elongation at different times of the day, we observed that GI function is required during the night period (Suppl. Fig. 11a) and its effect is photoperiod dependent, that is, the longer the dark period is, the greater is the observed difference in growth between *gi-2* and wildtype plants (Suppl. Fig. 11b). We hence hypothesized that GI function is required to properly phase growth rhythms towards the end of the night under SD photoperiods. Growth rhythm measurements in SDs revealed that *gi-2* mutant plants display a general growth de-repression, which is especially remarkable during the early night (Fig. 4a). Maximal growth was also observed to peak earlier (Fig. 4a), which might be linked to *gi-2* shorter period (Park et al. 1999, Mizoguchi et al. 2005, Martin-Tryon et al. 2007). Strikingly, loss of *PIF3* partially rescued not only the average growth rate phenotype, but also shifted the maximal growth phase to peak later (Fig. 4a; Suppl. Fig. 11c).

Earlier work suggested a role for PIF proteins in the circadian clock, and PIF3 was found to bind G-box containing sequences from the *CCA1* and *LHY* promoter regions (Martinez-Garcia et al. 2000). However, this implication could not be subsequently confirmed using *PIF3* single mutants and overexpression lines (Viczian et al. 2005), although the high level of redundancy among PIFs complicates the interpretation of these results. More recently, ChIP experiments followed by deep sequencing have revealed the binding of PIFs to the promoters of clock genes *in vivo* (Oh et al. 2012, Zhang et al., 2013), and the interaction between PIFs and several clock components has been reported (Nieto et al. 2015, Soy et al. 2016, Zhu et al. 2016). The effect of the *PIF3* mutation on the growth phase phenotype of *gi-2* mutants prompted us to investigate the relevance of the GI-PIF interaction for the regulation of circadian rhythmicity. Gene expression analyses implied that PIFs repress the expression of the core clock gene *CCA1* under SD conditions (Fig. 4b), an observation that was confirmed in transient activation assays in *N. benthamiana* leaves using the *pCCA1*::LUC reporter (Suppl. Fig. 12a). Notably, mutation of *PIF4* and *PIF5* modestly alleviated the very low level of *CCA1* expression in *gi-2* mutants (Fig. 4b), suggesting that excessive PIF activity in *gi-2* may be partially responsible for the low amplitude of *CCA1*. Analysis of *pCCA1* promoter activity in *Arabidopsis* seedlings under free running conditions revealed that, although *pif3-1* mutation does not show an obvious period phenotype (Suppl. Fig. 12b,c,d), *pif4-101;pif5-1* double mutants display a lengthened period (Fig. 4c, Suppl. Fig. 12e,f), confirming the role of PIF proteins in determining the pace of the clock. More importantly, loss of *PIF3* in the *gi-2* background increased the amplitude of *pCCA1* oscillations and lengthened the short period of *gi-2* mutants by 1 hour (Fig. 4d,e, Suppl. Fig. 12g). The double *gi-2;pif3-1* mutants also maintained rhythmicity for longer under free running conditions compared to *gi-2* seedlings, which displayed less robust oscillations with higher relative amplitude error (RAE) and became arrhythmic after the third day in constant light (Fig. 4d,e). The effect of *PIF3* mutation on ameliorating the rhythmicity of *gi-2* mutants was also observed by leaf movement analysis (Fig. 4f,g, Suppl. Fig. 12h). To this purpose, seedlings were entrained in SD conditions for 7 days and then released to constant light; under these free running conditions, cotyledon tip to tip distance was measured to assess rhythms in cotyledon movement. In our experimental setting, *gi-2* mutant seedlings turned out to be arrhythmic, displaying very low amplitude cotyledon movements for which no period could be calculated. In the case of *pif3-1* mutants, these were again indistinguishable from wildtype plants, but *pif3-1* mutation in the *gi-2* background partially restored rhythmicity, amplifying oscillations and allowing for period length calculation of *gi-2;pif3-1* plants, which displayed a short period as seen by bioluminescence imaging (Fig. 4f,g, Suppl. Fig. 12h).

Taken together, our results uncover a role of PIF proteins in the circadian system, directly linking light signaling to *CCA1* transcriptional regulation. We also show that modulation of PIF activity by GI is required to not only adequately phase output rhythms such as growth, but also for proper clock progression. Mutation of *PIF* genes only partially rescued *gi-2* circadian phenotypes, denoting the additional mechanisms that take place in the regulation of *CCA1* expression by GI and in GI function in the circadian system. Furthermore, although PIF proteins seem to repress *CCA1* and their mutation partially restores *CCA1* amplitude in *gi-2*, single and double *PIF* mutants show themselves low *pCCA1* amplitude (Suppl. Fig. 12b,e). This suggests a more complex regulatory scenario where PIFs, despite functioning as *CCA1* repressors, may also be required for normal activation of *CCA1* at dawn through yet to be identified mechanisms that may include, among others, recruitment of activators.

**Concluding Remarks**

Precise timing of physiological processes by the circadian clock provides an adaptive advantage for organisms (Millar 2016). In plants, many developmental processes, including photoperiodic growth, are tightly regulated by the circadian clock in conjunction with multiple signaling pathways, combining a variety of internal and external signals (Greenham & McClung 2015, Sanchez & Kay, 2016). Efficient decoding and integration of the multiple stimuli perceived is therefore fundamental for these complex regulatory networks to be robust and advantageous in the coordination of development.

Here we provide evidence for a mechanism directly linking a component of the circadian oscillator to the gating of light and hormone signaling (Fig. 4h). We show that GI accumulation during the day stabilizes the DELLA repressors and interferes with PIF DNA binding ability. We propose that GI binding to the DELLAs at the end of the day blocks the access of the GA receptor, which is highly expressed at this time of the day (Arana et al. 2011). In addition, GI prevents the binding of the PIFs to the chromatin, not only through direct interaction, but also by occupying their target promoter regions at dusk. Progressive degradation of GI during the early evening (David et al. 2006, Yu et al. 2008) enables the degradation of the DELLA proteins and the release of the PIFs, which in the absence of photoactivated phyrochrome are able to accumulate and access target promoter regions later in the night. We show how this mechanism is required to adequately phase growth rhythms in SD photoperiods, and, given the pivotal role of PIF transcription factors and DELLA proteins in plant development (Leivar & Quail 2011, Xu et al. 2014, Daviere & Achard 2016), we hypothesize that their circadian gating by GI is likely to have implications beyond the control of hypocotyl elongation, affecting a variety of physiological pathways that may include biotic and abiotic stress responses, floral induction, and root development. Finally, our results reveal the involvement of PIF proteins in clock progression through the regulation of *CCA1* transcription, and show the relevance of PIF activity modulation by GI in setting the pace of the clock.

Hence, our study assigns a role to GI as an integrator of external and internal signals to regulate both the circadian clock and its output, and provides a mechanistic framework to further investigate the circadian gating of plant development and how light signals are transmitted into the circadian system.

**Figure legends**

**Figure 1.** **GI shares targets with light signaling components, and interacts with PIF and DELLA proteins.** **a**, Venn diagram showing the overlap between DEGs in *gi-2* (Kim et al. 2012) and *pifQ* mutants (Oh et al., 2012) (p-value=5.225e-14). **b**, Phase diagram depicting the p-value of the phase of peak expression enrichment (count/expected) of genes differentially expressed in *gi-2* only (GI), *pifQ* only (PIFs), and in both *gi-2* and *pifQ* (GI-PIFs) under SD conditions. Day period is marked in yellow and night period in gray. Statistically significant phase enrichments (p-value<0.01) are marked with a star. **c**,**d**, *In vitro* pull-down assays showing the interaction between GI and PIFs (PIF1, PIF3, PIF4, and PIF5) (**c**) and GI and DELLAs (RGA, GAI, and RGL3) (**d**). Proteins were expressed in a TNT *in vitro* expression system and immunoprecipitated with either anti-HA (**c**) or anti-Flag antibodies (**d**). The recovered fractions were analyzed by Western blot using anti-Flag and anti-HA anitibodies. **e**, *In vivo* co-immunoprecipitation studies in *Arabidopsis* transgenic seedlings expressing GI-YPET-Flag and PIF3-ECFP-HA (left panel) or GI-YPET-Flag and PIF5-HA (right panel) tagged protein versions. Total protein extracts were immunoprecipitated with anti-Flag antibody and analyzed by Western blot using anti-Flag and anti-HA anitibodies. **f**, *In planta* co-immunoprecipitation analysis from *N. benthamiana* leaves expressing Flag-GI and HA-RGA. Total protein extracts were immunoprecipitated with anti-Flag antibody and analyzed by Western blot using anti-Flag and anti-HA anitibodies. **g**, Confocal imaging of *N. benthamiana* leaves expressing GI-YPET, RGA-mCherry, and PIF3-ECFP (upper panel) or GI-YPET, RGA-mCherry, and PIF5-ECFP (lower panel). Merged images of YPET, mCherry, and ECFP fluorescence show co-localization of GI, RGA, and PIF3/5 proteins to nuclear bodies. BF, bright field image. **c**-**g**, All experiments were repeated with similar results.

**Figure 2. GI stabilizes RGA by hindering GID1A access and gates GA sensitivity.** **a**, Accumulation of GFP-RGA across SD photo-cycles in wildtype (col0), *gi-2*, and GIox backgrounds. Seedlings were grown for 10 days under SD conditions and samples were collected every 4 hours. Protein levels were determined by Western blot analysis with anti-GFP antibody and normalized against actin levels. The quantitation of 3 biological replicates is shown. Values represent mean ± SEM. **b**, Scatter plot of hypocotyl length measurements from wildtype (col0), GIox, *rga-29;gai-td1*, and GIox*;rga-29;**gai-td1* seedlings grown for 7 days in SDs (n=24-36). **c**, Analysis of the interaction between GID1A and RGA in the absence and presence of increasing quantities of GI (0.25x, 0.5x, 1x, 2x, and 4x) through *in vitro* pull-downs and Western blot (upper panel). The quantitation of the relative amount of RGA co-immunoprecipitated with GID1A in every fraction is shown in the lower panel. **d**, Degradation time-course of GFP-RGA in wildtype (col0) and *gi-2* mutants incubated with 100 µM GA3. Protein levels were determined by Western blot with anti-GFP antibody and normalized against actin levels. The quantitation of the relative amount of GFP-RGA in every fraction is shown in the lower panel. The experiment was repeated with similar results. **e**, Hypocotyl length increase of seedlings grown for 5 days under SD conditions in the presence of 0.2 µM PAC and treated with 1 µM GA4 at different ZTs (mean ± SEM, n=25).

**Figure 3. GI represses PIF activity and hinders their access to target chromatin.** **a**, Expression analysis by RT-qPCR of *PIL1* and *HFR1* in wildtype (col0), *gi-2*, and GIox seedlings grown for 10 days in SDs. White and gray shadings represent day and night, respectively. Expression levels are relative to the expression of *PP2A*. Values represent mean ± SEM of 3 biological replicates. **b**, Transactivation assays in *N. benthamiana* leaves. Different effectors were co-expressed with the *pPIL1*::LUC reporter construct. Luminescence was measured 2 days post-infiltration in SD conditions. Results show mean ± SEM (n=12). **c**, ChIP assays of 10-day-old seedlings grown in SD conditions and harvested at ZT16. Relative fold enrichment over actin in the different fragments was quantified by qPCR. The experiment was repeated twice with similar results. **d**, ChIP assays of 10-day-old seedlings grown in SD conditions and harvested at ZT8 and ZT16. Relative fold enrichment over *PP2A* in the different fragments was quantified by qPCR. The experiment was repeated twice with similar results. **e**, Expression analysis by RT-qPCR of *PIL1* and *HFR1* in wildtype (col0), *gi-2*, *pif3-1*, and *gi-2;pif3-1* seedlings grown for 3 days in SDs. White and gray shadings represent day and night, respectively. Expression levels are relative to the expression of *PP2A*. Values represent mean ± SEM of 3 biological replicates. **f**, Expression analysis by RT-qPCR of *PIL1* in wildtype (col0), *gi-2*, *gi-2;pif3-1*, *gi-2;pif4-101;pif5-1*, and *pifQ* seedlings grown for 7 days in SDs. White and gray shadings represent day and night, respectively. Expression levels are relative to the expression of *PP2A*. Values represent mean ± SEM (n=3). The experiment was repeated twice with similar results. **g**, Scatter plot of hypocotyl length measurements from wildtype (col0), *gi-2*, *pif3-1*, *pif4-101;pif5-1*, *gi-2;pif3-1*, *gi-2;pif4-101*, *gi-2;pif5-1*, *gi-2;pif4-101;pif5-1*, and *pifQ* seedlings grown for 2 weeks in SDs (n=9-20).

**Figure 4. GI modulation of light signaling affects output and circadian rhythms.** **a**, Growth rate measurements (mm/h) of wildtype (col0), *gi-2*, *pif3-1*, and *gi-2;pif3-1* seedlings grown in SDs. Values represent mean ± SEM (n=). Time is expressed in hours after stratification. White and gray shadings represent day and night, respectively. **b**, Expression analysis by RT-qPCR of *PIL1* in wildtype (col0), *gi-2*, *gi-2;pif3-1*, *gi-2;pif4-101;pif5-1*, and *pifQ* seedlings grown for 7 days in SDs. White and gray shadings represent day and night, respectively. Expression levels are relative to the expression of *PP2A*. Values represent mean ± SEM (n=3). **c**, Period length estimations versus relative amplitude error (RAE) of *pCCA1*::LUC reporter expression in wildtype (col0) and *pif4-101;pif5-1* mutant seedlings as analyzed by FTT-NLLS (n=12). **d**, Bioluminescence analysis of *pCCA1*::LUC in wildtype (col0), *gi-2*, and *gi-2;pif3-1* seedlings in constant light (LL). Values represent mean ± SEM (n=12). Plants were entrained in SDs for 7 days; the upper white and gray bars represent subjective day and night, respectively. **e**, Period length estimations versus RAE of *pCCA1*::LUC in wildtype (col0), *gi-2*, and *gi-2;pif3-1* as analyzed by FTT-NLLS. **f**, Cotyledon movement analysis of wildtype (col0), *gi-2*, *pif3-1*, and *gi-2;pif3-1* seedlings in constant light (LL). Values represent mean ± SEM (n=4-5). Plants were entrained in SDs for 7 days; the upper white and gray bars represent subjective day and night, respectively. **g**, Period length estimations versus RAE of wildtype (col0), *pif3-1*, and *gi-2;pif3-1* cotyledon movements as analyzed by FTT-NLLS. No period length could be estimated for *gi-2* seedlings. **b**-**g**, All experiments were repeated with similar results. **h**, Model depicting the function of GI in the circadian gating of hormone and light signaling pathways. As GI accumulates during the day, it stabilizes the DELLAs by hindering access of the GA receptor GID1A, whose expression is circadianly regulated and high in the evening. In addition, GI prevents the binding of the PIFs to the chromatin through direct interaction and competition for target promoter regions at dusk. Progressive degradation of GI during the early evening enables the degradation of the DELLA proteins and the release of the PIFs, which in the absence of photoactivated phyrochrome are able to accumulate and access target promoter regions later in the night. The proposed mechanism is required not only to adequately phase output rhythms such as growth, but also to set the pace of the clock.

**Supplementary figure legends**

**Supplementary figure 1.** **GI shares targets with both clock and light signaling pathways. a**, Over-represented *cis* elements at the promoter regions of DEGs in *gi-2* seedlings (Kim et al. 2012). **b**, Venn diagram showing the overlap between DEGs in *gi-2*, *pifQ* (Oh et al., 2012), and *cca1-1;lhy-11* mutants (Kamioka et al., 2016). **c**, Heat map showing the GO term enrichment scores of genes differentially expressed in *gi-2* only (GI), in both *gi-2* and *pifQ* (GI-PIFs), and in both *gi-2* and *cca1-1;lhy-11* (GI-CCA1/LHY). **d**,**e**, Phase diagrams depicting the p-value of the phase of peak expression enrichment of genes differentially expressed in *gi-2* only (GI), *pifQ* only (PIFs), and in both *gi-2* and *pifQ* (GI-PIFs) (**d**) or in *gi-2* only (GI), *cca1-1;lhy-11* only (CCA1;LHY), and in both *gi-2* and *cca1-1;lhy-11* (GI/CCA1;LHY) (**e**) under 12h light and 12h dark conditions. Day period is marked in yellow and night period in gray. Statistically significant enrichments (p-value<0.01) are marked with a star.

**Supplementary figure 2.** ***gi* mutant plants display a long hypocotyl phenotype under different light conditions.** Scatter plot of hypocotyl length measurements from wildtype (col0) and *gi-2* seedlings grown for 6 days under different light conditions (n=20-36). D, darkness; cR, constant red light (1 µmol m2s-1); cB, constant blue light (1 µmol m2s-1); SD, short day photoperiod (8h light, 16 h dark).

**Supplementary figure 3.** **GI interacts with PIF and DELLA proteins in yeast.** Yeast two-hybrid (Y2H) assays showing interaction of GI and PIF proteins (**a**,**c**), and GI and DELLA proteins (**b**,**c**). Bait and prey constructs were co-transformed into yeast cells. SD-WL, minimal medium lacking Trp and Leu; SD-WLH, selective medium lacking Trp, Leu and His, which was supplemented with 50 mM 3AT; X-gal, qualitative β-galactosidase activity results obtained for the X-gal assay. **c**, Quantitation of β-galactosidase activity (Miller units) for every pair of bait and prey proteins indicated (n=4). Values represent means ± SEM. All Y2H analyses were repeated with similar results.

**Supplementary figure 4.** **GI interacts with PIF3 bHLH domain and RGA GRAS domain.** *In vitro* pull-down assays performed to map the interaction domains between GI and PIF3 (**a**) and GI and RGA (**b**). Proteins were expressed in a TNT *in vitro* expression system and immunoprecipitated with anti-HA antibody. The recovered fractions were analyzed by Western blot using anti-Flag and anti-HA anitibodies. A scheme of the deleted protein versions is shown on the upper panels. All analyses were repeated with similar results.

**Supplementary figure 5.** **GI co-localizes with PIF3/5 and RGA in the nucleus.** Confocal microscopy images showing co-localization of GI and PIF3, GI and PIF5, or GI and RGA. Imaging was performed on *N. benthamiana* leaves expressing GI fused to YPET and PIF3/5 fused to ECFP (upper panels) or GI fused to YPET and RGA fused to mCherry (lower panel). BF, bright field image. The experiment was repeated with similar results.

**Supplementary figure 6. GI promotes RGA stability. a**, Representative Western blot showing the accumulation of RGA-GFP in *N. benthamiana* leaves treated with 25 µM MG-132 or in the presence or absence of GI-HA. Protein levels were determined by Western blot analysis and normalized against HA-GFP levels. The quantitation of 3 biological replicates is shown on the right. Values represent mean ± SEM. **b**, Representative Western blot showing the accumulation of GFP-RGA across SD photo-cycles in wildtype (col0), *gi-2*, and GIox backgrounds. Protein levels were determined with anti-GFP antibody and actin levels were used for normalization. The right panel shows representative confocal images taken from all lines at ZT20.

**Supplementary figure 7.** **GI stabilizes RGA in the context of the GA-GID1A pathway by hindering GID1A access to RGA. a**, *In vitro* pull-down study of the interaction between Flag-GID1A and HA-RGA in the presence and absence of cMyc-GI. Proteins were expressed in a TNT *in vitro* expression system and immunoprecipitated with anti-Flag antibody. The recovered fractions were analyzed by Western blot using anti-Flag, anti-HA, and anti-cMyc antibodies. **b**, Degradation time-course of GFP-RGA in wildtype (col0) and *gi-2* mutants incubated with 100 µM GA3 and 50 uM MG-132. Protein levels were determined by Western blot analysis with anti-GFP antibody and normalized against actin levels. The quantitation of the relative amount of GFP-RGA in every fraction is shown in the lower panel. **c**, RG52-GFP accumulation in *N. benthamiana* leaves treated with 25 µM MG-132 or in the presence of GI-HA. Protein levels were determined by Western blot analysis and normalized against HA-GFP levels. Western blot quantitation is shown on the lower panel. Values represent mean ± SEM (n=3). **d**, Scatter plots of hypocotyl length measurements from *pRGA*::GFP-RGA lines in wildtype (col0), *gi-2*, and GIox backgrounds and their respective control lines grown for 10 days in SDs (left panel, n=16-22) and wildtype (Ler), *gi-3*, *gai-1D*, and *gi-3;gai-1D* seedlings grown for 7 days in SDs (right panel, n=16-20).

**Supplementary figure 8.** **GI affects GA signaling through DELLA protein stabilization. a**, GA3 and PAC dose response curves for wildtype (col0) and *gi-2* mutant seedlings. Plants were grown for 7 days under SD conditions with increasing concentrations of GA3 (0, 0.1, 1, and 10 µM, left panel) or for 3 days in the dark in the presence of increasing concentrations of PAC (0, 0.02, 0.2, and 2 µM, right panel). Values represent means ± SEM (n=24-36). **b**, Expression analysis by RT-qPCR of *RGA*, *GAI*, *GID1A*, and *GA20ox2* in wildtype (col0), *gi-2*, and GIox seedlings grown for 10 days in SDs. White and gray shadings represent day and night, respectively. Expression levels are relative to the expression of *PP2A*. Values represent mean ± SEM of 3 biological replicates. **c**, Hypocotyl length of *HS*::gai-1D lines in wildtype (col0), *gi-2* and GIox backgrounds treated with heat at ZT12 to induce the expression of the GAI dominant negative version gai-1D (brown bars) compared to non-treated controls (gray bars). Values represent mean ± SEM, n=33-39.

**Supplementary figure 9.** **GI affects the expression of PIF target genes, but does not greatly contribute to *PIF* mRNA expression and protein accumulation.** **a**,**b**, Expression analysis by RT-qPCR of *IAA19* and *PRE5* (**a**) and *PIF1*, *PIF3*, *PIF4*, and *PIF5* (**b**) in wildtype (col0), *gi-2*, and GIox seedlings grown for 10 days in SDs. White and gray shadings represent day and night, respectively. Expression levels are relative to the expression of *PP2A*. Values represent mean ± SEM of 3 biological replicates. **c**, PIF3-ECFP-HA accumulation in *N. benthamiana* leaves treated with 25 µM MG-132 or in the presence of GI-HA. Protein levels were determined by Western blot analysis and normalized against HA-GFP levels. Western blot quantitation is shown on the lower panel. Values represent mean ± SEM (n=3).

**Supplementary figure 10.** **GI represses PIF transcriptional activity. a**,**b**, Transactivation assays in *N. benthamiana* leaves. Different effectors were co-expressed with the *pPIL1*::LUC reporter construct. Luminescence was measured 2 days post-infiltration in SD conditions. Results show mean ± SEM (**a**, n=12; **b**, n=8). **c**, Western blot analysis of the expression of HA-GI and HA-RGA in the transactivation experiment shown in Figure 1b. **d**, Western blot analysis of the levels of PIF3-ECFP-HA at ZT16 in the lines used for the ChIP analysis shown in Figure 1c. For detection, anti-HA antibody was used and 3 replicates were analyzed for each line. The quantitation of the 3 replicates relative to actin is shown on the lower panel. Values represent mean ± SEM.

**Supplementary figure 11. GI function is required during the night period and its effect is photoperiod dependent. a**, Hypocotyl length of SD grown wildtype (col0) and *gi-2* seedlings measured at different times after stratification. Values represent mean ± SEM (n=31-49). **b**, Scatter plot of hypocotyl length measurements from wildtype (col0) and *gi-2* seedlings grown for 10 days under different photoperiods (n=20-33). LD, long day (16h light, 8h dark); 12/12, 12h light/12h dark; SD, short day (8h light, 16 h dark). **c**, Overlap of the growth rate measurement traces (mm/h) of *gi-2* and *gi-2;pif3-1* seedlings shown in Figure 4a. Time is expressed in hours after stratification. White and gray shadings represent day and night, respectively.

**Supplementary figure 12. PIFS affect clock function by repressing *CCA1* and their regulation by GI is required for proper clock progression.** **a**, Transactivation assays in *N. benthamiana* leaves. Different effectors were co-expressed with the *pCCA1*::LUC reporter construct. Luminescence was measured 2 days post-infiltration in SD conditions. Results show mean ± SEM (n=8). **b**, Bioluminescence analysis of *pCCA1*::LUC in wildtype (col0) and *pif3-1* seedlings in constant light (LL). Values represent mean ± SEM (n=24). Plants were entrained in SDs for 7 days; the upper white and gray bars represent subjective day and night, respectively. **c**, Period length estimations versus RAE of *pCCA1*::LUC in wildtype (col0) and *pif3-1* as analyzed by FTT-NLLS. **d**, Scatter plot of the periods estimated for wildtype (col0) (25.52 ± 0.2 h, n=21) and *pif3-1* (26.07 ± 0.17 h, n=19).The error bars represent ± SEM. **e**, Bioluminescence analysis of *pCCA1*::LUC in wildtype (col0) and *pif4-101;pif5-1* seedlings in constant light (LL). Values represent mean ± SEM (n=12). Plants were entrained in SDs for 7 days; the upper white and gray bars represent subjective day and night, respectively. **f**, Scatter plot of the periods estimated for wildtype (col0) (24.93 ± 0.25 h, n=12) and *pif4-101;pif5-1* (26.17 ± 0.07 h, n=12).The error bars represent ± SEM. **g**, Scatter plot of the *pCCA1*::LUC expression period estimated for wildtype (col0) (24.07 ± 0.19 h, n=12), *gi-2* (21.51 ± 0.08 h, n=11), and *gi-2;pif3* (22.52 ± 0.18 h, n=12).The error bars represent ± SEM. **h**, Scatter plot of the periods estimated for wildtype (col0) (25.31 ± 0.24 h, n=5), pif3-1 (25.69 ± 0.13 h, n=5), and gi-2;pif3-1 (21.28 ± 0.11 h, n=4) cotyledon movements. The error bars represent ± SEM. **a**-**h**, All experiments were repeated with similar results.