the plant journal

SEB Society for Experimental Biology

The Plant Journal (2015) **81,** 695–706 doi: 10.1111/tpj.12759

The GI–CDF module of Arabidopsis affects freezing tolerance and growth as well as flowering

Fabio Fornara[1,2], Amaury de Montaigu[1], Alfredo Sánchez-Villarreal[1], Yasuyuki Takahashi[1], Emiel Ver Loren van Themaat[1], Bruno Huettel[1], Seth J. Davis[1] and George Coupland[1,*]

[1]*Max Planck Institute for Plant Breeding Research, Carl von Linné Weg 10, D–50829 Cologne, Germany, and*
[2]*Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy*

Received 24 July 2014; revised 28 November 2014; accepted 22 December 2014; published online 19 January 2015.
*For correspondence (e-mail coupland@mpipz.mpg.de).

SUMMARY

Plants monitor and integrate temperature, photoperiod and light quality signals to respond to continuous changes in their environment. The GIGANTEA (GI) protein is central in diverse signaling pathways, including photoperiodic, sugar and light signaling pathways, stress responses and circadian clock regulation. Previously, GI was shown to activate expression of the key floral regulators *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) by facilitating degradation of a family of CYCLING DOF FACTOR (CDF) transcriptional repressors. However, whether CDFs are implicated in other processes affected by GI remains unclear. We investigated the contribution of the GI–CDF module to traits that depend on GI. Transcriptome profiling indicated that mutations in *GI* and the *CDF* genes have antagonistic effects on expression of a wider set of genes than *CO* and *FT*, whilst other genes are regulated by GI independently of the CDFs. Detailed expression studies followed by phenotypic assays showed that the CDFs function downstream of GI, influencing responses to freezing temperatures and growth, but are not necessary for proper clock function. Thus GI-mediated regulation of CDFs contributes to several processes in addition to flowering, but is not implicated in all of the traits influenced by GI.

Keywords: *GIGANTEA*, *CYCLING DOF FACTOR*, flowering, freezing tolerance, growth, circadian clock, *Arabidopsis thaliana*.

INTRODUCTION

Environmental cues drive developmental switches, including flowering, and affect physiological responses, such as growth or tolerance to stresses. These external inputs are coordinated with endogenous regulatory networks to adapt plant growth and development to the environment. The *GIGANTEA* (*GI*) locus in Arabidopsis was originally identified because mutations in the gene prevented a flowering response to inductive photoperiods (Redei, 1962; Koornneef *et al.*, 1991; Fowler *et al.*, 1999; Park *et al.*, 1999;). However, *GI* also mediates responses to environmental cues, including light and abiotic stresses, and affects gene expression by contributing to the activity of the endogenous circadian oscillator (Kurepa *et al.*, 1998; Huq *et al.*, 2000; Cao *et al.*, 2005; Dalchau *et al.*, 2011; Kim *et al.*, 2012, 2013a; Riboni *et al.*, 2013).

GI has been best characterized for its role in photoperiodic flowering, where it is required to promote transcription of *CONSTANS* (*CO*), which encodes a zinc finger transcription factor, and *FLOWERING LOCUS T* (*FT*), a florigenic protein sharing similarity with RAF-kinase inhibitors (Fornara *et al.*, 2010). Activation of *CO* and *FT* expression under long days depends on the degradation of repressors belonging to the DOF family of transcriptional regulators, collectively known as CYCLING DOF FACTORS (CDFs), which include CDF1, CDF2, CDF3 and CDF5 (Imaizumi *et al.*, 2005; Fornara *et al.*, 2009). A protein complex formed by GI and an F–box protein encoded by *FLAVIN BINDING KELCH REPEAT F–BOX PROTEIN 1* (*FKF1*) is required to degrade the CDF proteins, thus releasing repression of *CO* and *FT* transcription (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). FKF1 homologs, including ZEITLUPE (ZTL) and LOV KELCH PROTEIN 2 (LKP2), act redundantly with FKF1 to reduce the amount of at least one CDF protein, and are required for proper progression and robustness of the circadian clock (Somers *et al.*, 2000; Kim *et al.*, 2007; Fornara *et al.*, 2009; Baudry *et al.*, 2010). GI, FKF1 and CDF1 associate with chromatin at the *CO* and *FT* loci, indicating that transcriptional regulatory mechanisms

occur directly at the promoters of these genes (Sawa *et al.*, 2007; Sawa and Kay, 2011; Song *et al.*, 2012).

Under temperate climates, long-day plants such as Arabidopsis flower during the spring or early summer, when the temperature is permissive. As days become shorter and the temperature decreases, they undergo cold acclimation, a process that involves extensive physiological and biochemical changes that are necessary to protect cells from low winter temperatures (Miura and Furumoto, 2013). Cold responses are under photoperiodic control, so that they are activated under short days (SDs) but not long days (LDs) (Lee and Thomashow, 2012). In Arabidopsis, exposure to low temperatures triggers rapid activation of cold-response genes, including *CBF1*/*DROUGHT RESPONSE ELEMENT BINDING FACTOR 1B* (*DREB1B*), *CBF2*/*DREB1C* and *CBF3*/*DREB1A*, which encode transcription factors of the AP2/ERF family (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Maibam *et al.*, 2013). The CBF proteins bind the *CRT*/*DRE* regulatory element, which is present in the promoter of several cold-regulated genes, and promote their expression. This regulatory module is known as the CBF regulon, and promotes freezing tolerance by inducing accumulation of low-molecular-weight osmoprotectants such as sucrose, raffinose, proline and cryoprotecting proteins (Gilmour *et al.*, 2000). Transcription of the *CBF* genes and their targets is under circadian and photoperiodic control (Fowler *et al.*, 2005; Covington *et al.*, 2008; Dong *et al.*, 2011; Lee and Thomashow, 2012), and light quality information mediated by red/far-red light photoreceptors also modulates transcription of *CBF* loci independently of temperature (Franklin and Whitelam, 2007).

Growth is also strongly dependent on endogenous and environmental signals, and is promoted through the activity of PHYTOCHROME INTERACTING FACTOR (PIF) transcription factors that integrate several regulatory pathways (Leivar and Monte, 2014; de Lucas and Prat, 2014). A combination of transcriptional and post-translational events ensures that two such proteins, PIF4 and PIF5, accumulate only at the end of the night, when growth rates are highest (Nozue *et al.*, 2007). The circadian clock controls cycling of *PIF4* and *PIF5* transcripts, whose expression is highest during the day and reaches a trough during the first part of the night (Nozue *et al.*, 2007). PhyB directly interacts with PIF4 and PIF5 proteins, targeting them for degradation in the light (Huq and Quail, 2002), reducing growth rates and ensuring that sufficient protein accumulates only during the night. As a consequence, Arabidopsis plants grown under SDs display longer hypocotyls due to high PIF accumulation and enhanced growth rates (Niwa *et al.*, 2009). Finally, DELLA repressors directly interact with PIFs, preventing their binding to DNA; however, as gibberellin levels rise, DELLA proteins are degraded through the proteasome, and PIFs are released to bind to the promoters of target genes (Feng *et al.*, 2008; de Lucas *et al.*, 2008). Plants bearing non-functional *GI* alleles have longer hypocotyls under red light, show enhanced growth rates, and express *PIF4* mRNA at higher levels (Huq *et al.*, 2000; Nozue *et al.*, 2007; Kim *et al.*, 2012). These data indicate that GI may act as a point of convergence of signaling pathways regulating growth, but the functional mechanisms involved in such processes are currently unknown.

To understand how the CDFs influence various aspects of the *gi* phenotype, and to identify novel targets of photoperiodic regulators, we generated and compared transcriptome profiles of *gi–100* (referred to as *gi* below), *cdf1–R cdf2–1 cdf3–1 cdf5–1* (referred to as *cdf1235* below) and *gi–100 cdf1–R cdf2–1 cdf3–1 cdf5–1* mutants (referred to as *gi cdf1235* below). We identified a set of genes whose transcription depends on the GI–CDF module, but also genes that are differentially expressed only in *gi* or *cdf1235* mutants. Our data indicate that regulation of genes involved in cold and stress responses depends on the GI–CDF module, through regulation of the CBF regulon. We provide genetic evidence to support a role for the module upstream of *PIF4* and *PIF5* in the control of growth. Finally, we show that clock rhythmicity is influenced by GI independently of the CDFs, indicating that components of the module have separable functions and that not all processes dependent on GI involve the CDFs.

RESULTS

GI and the CDFs control transcription of genes related to light signaling, circadian clock function, flowering and stress responses

A microarray approach was used to identify the genes regulated by GI and the CDFs. In this section, each genotype is considered separately. Using AGRONOMICS1 arrays, the transcriptomes of Col–0, the *gi* mutant, the *cdf1235* quadruple mutant and the *gi cdf1235* quintuple mutant were analyzed at ZT12 when GI protein abundance is highest (David *et al.*, 2006; Fornara *et al.*, 2009; Rehrauer *et al.*, 2010). A total of 199 differentially expressed genes were identified by comparing Col–0 and *gi* (Figure 1a). Of these, 161 genes were up-regulated in the mutant and 38 were down-regulated (Data S1 and Table S1). Among the differentially expressed genes, 76 (38%) and 57 (29%) were previously reported as differentially expressed in two studies that profiled mutants carrying the *gi–2* allele (Kim *et al.*, 2012, 2013b).

Thirty-six genes were up-regulated in *cdf1235* mutants compared to Col–0, including *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FRUITFULL* (*FUL*). Additionally, 50 genes were down-regulated. These data indicate that CDFs also increase transcription of downstream genes, although it is not clear whether any of these are direct targets (Data S1 and Table S2). Altered

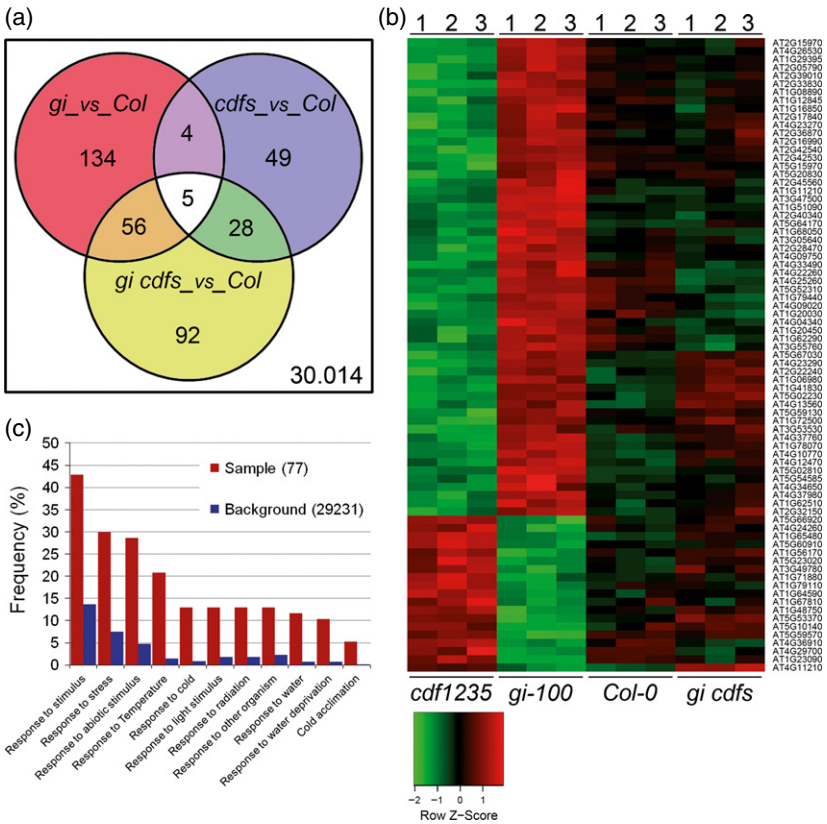

Figure 1. Effects of GIGANTEA and CDF transcription factors on global gene expression.
(a) Venn diagrams showing overlap between genes transcriptionally regulated by GI, CDF1235 and GI CDF1235.
(b) Heat map showing clustering of genes that show opposite patterns of regulation in *gi* and *cdf1235* mutants, and whose expression is restored to wild-type levels in *gi cdf1235*. Red and green indicate increased and decreased expression, respectively, compared to mean expression across all arrays. The numbers on the top of the columns indicate biological replicates. The Arabidopsis Genome Initiative identifier is shown on the right. Note that *CO* is not included in this map because the results were not statistically significant in the comparison Col–0 versus *cdf1235*.
(c) GO category enrichment for genes listed in (b). The frequency in the sample is plotted, together with the frequency observed in the Arabidopsis genome (background).

expression of several downstream genes was further validated in independent time courses, which supported the array data across 24 h diurnal cycles (Figure S1).

There was a significant overlap between genes regulated by GI and those whose transcription is altered by stress treatments, including cold, drought and abscisic acid (Table S3) (Covington *et al.*, 2008; Matsui *et al.*, 2008), whereas the *cdf1235* dataset was enriched in cold- and abscisic acid-responsive genes (Table S3). Diurnally or clock-controlled genes showed a significant overlap with the *gi* dataset, but no statistically significant overlap was observed for genes regulated by *CDF1235* (Table S3). Phase enrichment measurements indicated that GI affects expression of genes that peak before (ZT3–ZT10) or after (ZT15 and ZT20) GI protein peak time (Figure S2). The data show that most genes whose phase of expression occurred earlier than GI protein peak time were repressed by GI, whereas genes showing later phases of expression almost exclusively comprised genes activated by GI, consistent with a lag between the highest accumulation of GI and transcriptional effects on downstream genes. The earlier phase of expression of repressed genes suggests that these may be indirectly regulated by GI (Figure S2). These data showed that GI and the CDFs have a broad impact on expression of genes involved in several regulatory processes other than flowering, particularly light signaling and stress responses, and influence gene expression at various times of day that do not coincide with the highest abundance of GI protein.

Antagonism of GI and CDF1235 on transcription of common downstream genes

Mutations in *GI* increase CDFs abundance, preventing induction of *CO* and *FT* and causing late flowering under LDs. The *gi cdf1235* quintuple mutant suppresses late flowering of *gi* mutants, restoring photoperiod-dependent flowering, normal *CO* and *FT* rhythmicity, and light responsiveness (Fornara *et al.*, 2009). Thus, *cdf* mutations are epistatic to *gi* in the regulation of *CO* and *FT* transcription.

Interpretation of Venn diagrams such as that shown in Figure 1(a) may be too conservative a method for detecting commonly de-regulated genes (Deng *et al.*, 2008). Therefore, to determine whether other genes were regulated by the GI–CDF module similarly to *CO*, a linear model with higher statistical sensitivity was developed to interrogate the datasets of differentially expressed genes in *gi*, *cdf1235* and *gi cdf1235*. All genes that showed opposing expression profiles in *gi* versus *cdf1235*, and whose expression was restored to levels similar to Col–0 in *gi cdf1235*, were extracted from the datasets. Of 77 genes that showed such patterns of transcription, 19 were identified as showing up-regulated transcription in *cdf1235* and

down-regulated transcription in *gi*, as previously shown for *CO* and *FT* (Figure 1b and Table S4). By contrast, the remaining 58 genes showed increased mRNA levels in *gi* and reduced abundance in *cdf1235* (Figure 1b and Table S4). Among this latter group of genes were several involved in drought and cold stress responses, including *EARLY RESPONSIVE TO DEHYDRATION* genes (*ERD10*, *ERD7* and *AT4G04340*) and cold-regulated genes (*COR78*, *KIN2*, *COR15a*, *COR15b*, *COR314* and *COR413*) (Kim and Nam, 2010; Thomashow, 2010). Gene clustering from the linear model dataset indicated that genes involved in responses to low temperatures and water deprivation were over-represented (Figure 1c).

Thus, the GI–CDF module controls transcription of a larger set of genes in Arabidopsis than simply the flowering-time genes *CO* and *FT*, and expression of such genes may either be promoted or repressed by GI.

cdf1235 mutations suppress the increased resistance to cold and oxidative stress of gi mutant plants

Exposure of Arabidopsis to low temperatures causes rapid activation of cold-responsive genes (Fowler and Thomashow, 2002), many of which were identified as co-regulated by GI and the CDFs (Figure 1b and Table S4). All genes identified showed increased mRNA levels in *gi* mutants, suggesting that GI represses expression of genes induced by cold stress. First, we determined the diurnal profiles of *COR15a* and *KIN1* mRNA, using samples collected over a 24 h time course (Figure 2a,c). Their phase of expression did not change between genotypes, but their amplitude of expression was enhanced in *gi* and strongly reduced in *cdf1235*. Expression levels were similar between Col–0 and *gi cdf1235*, indicating that *cdf1235* mutations suppress the increased mRNA expression of *COR15a* and *KIN1* observed in *gi*. After 10 days of growth under LDs, the *gi*, *cdf1235* and *gi cdf1235* mutants reached different developmental stages, with *cdf1235* seedlings undergoing floral transition while *gi* and *gi cdf1235* were still at the vegetative stage. To control for the influence of differing developmental stages on gene expression, 10-day-old seedlings grown under SDs, when all plants were still at the vegetative stage, were analyzed, and similar differences to those observed under LDs were detected between genotypes for both *COR15a* (Figure 2b) and *KIN1* (Figure 2d). These results suggest that cold-responsive genes are up-regulated in *gi* mutants due to increased expression of CDF1235.

We next measured responses to low temperatures by exposing seedlings grown under LDs to freezing

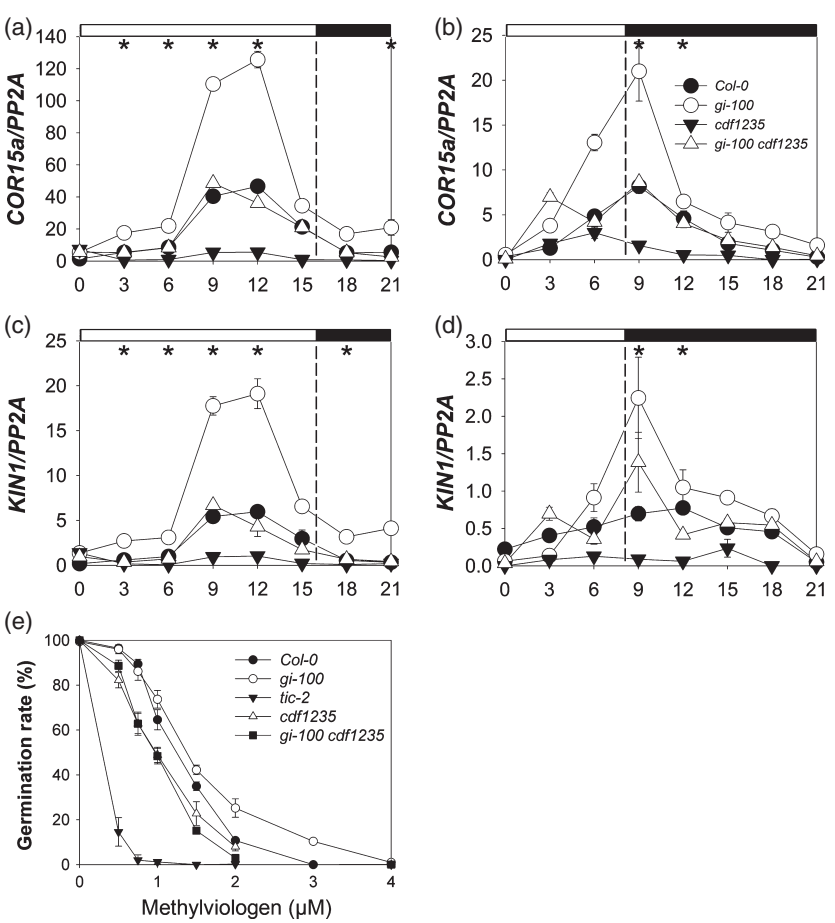

Figure 2. GIGANTEA and CDF transcription factors affect freezing tolerance.
(a) Diurnal expression of *COR15a* mRNA under LDs.
(b) Diurnal expression of *COR15a* mRNA under SDs.
(c) Diurnal expression of *KIN1* mRNA under LDs.
(d) Diurnal expression of *KIN1* mRNA under SDs.
Gene expression was normalized using *PROTEIN PHOSPHATASE 2A* (*PP2A*). The white and black bars on the top of each graph indicate day and night periods, respectively. Error bars indicate standard deviation of three technical replicates. The *P* values were determined by ANOVA ($P \leq 0.05$) using data from three consecutive time points in a sliding-window approach (Faure *et al.*, 2012). Asterisks indicate that gene expression was significantly induced or repressed by *gi* and *cdf1235* mutations, respectively, in agreement with the results of the microarray. Numbers on the *x* axis indicate hours from light on (ZT, Zeitgeber time).
(e) Tolerance to oxidative stress of Col–0, *gi*, *cdf1235*, *gi cdf1235* and *tic–2* mutants. The germination rates of seedlings grown on plates supplemented with increasing concentrations of methylviologen are plotted.

temperatures, with and without prior cold acclimation. The survival rate of *gi* seedlings was always higher than that of Col–0 plants, positively correlating with higher mRNA levels of cold tolerance genes (Table 1). The *cdf1235* mutations reduced the resistance of *gi* seedlings to cold, and similar effects were detected when plants were cold-acclimated (Table 1). The *cdf1235* mutations therefore reduced the resistance of *gi* seedlings to cold.

Freezing temperatures cause cell dehydration and membrane damage, partly through production of reactive oxygen species (Baek and Skinner, 2012). Several *gi* mutant alleles caused increased resistance to oxidative damage induced by methylviologen (also called paraquat) or H_2O_2 (Kurepa *et al.*, 1998). Therefore, the germination rates of *gi*, *cdf1235*, *gi cdf1235* and Col–0 were tested on increasing concentrations of methylviologen, and the *tic–2* mutant, which is hyper-sensitive to oxidative damage (Sanchez-Villarreal *et al.*, 2013), was included as a positive control. These experiments confirmed that *gi* mutants are more resistant to oxidative stress induced by methylviologen, and demonstrated that *cdf1235* mutants showed higher susceptibility to methylviologen compared to Col–0, whilst *cdf1235* suppressed the higher resistance shown by *gi* mutants (Figure 2e).

Protection from cold stress is partly achieved by accumulating osmoprotectants, including galactinol, raffinose and proline, which also show antioxidant properties (Kavi Kishor *et al.*, 1995; Taji *et al.*, 2002; Nishizawa *et al.*, 2008). Array data indicated that *ARABIDOPSIS THALIANA GALACTINOL SYNTHASE 3* (*AtGolS3*) and Δ^1*–PYRROLINE-5–CARBOXYLATE SYNTHASE 1* (*P5CS1*), which encode major enzymes involved in the biosynthesis of galactinol and proline, respectively, were up-regulated in *gi* compared to Col–0. We conclude that *gi* mutants display features of cold-acclimated plants, without having previously been exposed to low temperatures, and this is probably due to increased levels of CDF1235, as mutations in these factors reduce the freezing and oxidative stress tolerance of *gi* mutants and expression of cold-responsive genes.

The *GI–CDF* module controls growth through PIF4 and PIF5

Under particular light regimes, *gi* mutants exhibit longer hypocotyls compared to wild-type plants (Huq *et al.*, 2000; Mizoguchi *et al.*, 2005; Nozue *et al.*, 2007). Enhanced hypocotyl growth of *gi* mutant seedlings was completely suppressed by mutations in CDF transcription factors when plants were grown under SDs (Figure 3a,b). Hypocotyl elongation was measured under various light conditions, and fluence response curves were determined for all genotypes. As previously reported, *gi* seedlings were hyporesponsive to growth inhibition by red light (Huq *et al.*, 2000), whereas *gi cdf1235* and *cdf1235* responded similarly to wild-type or were hyper-responsive to exposure to red light, respectively (Figure 3c). No differences in hypocotyl elongation between genotypes were detected when seedlings were grown under far-red light (Figure 3d). These data indicate that CDF transcription factors promote hypocotyl growth, and this effect is antagonized by GI. Additionally, the GI–CDF regulatory module affects growth under red light but not far-red light, indicating dependence on PhyB signaling.

PIF4 and *PIF5* mRNA levels were increased in *gi* mutants (Table S1) (Kim *et al.*, 2012), but no statistically significant change in expression was detected in the *cdf1235* array data at ZT12. Growth rates are highest at the end of the night, because of coincidence between the clock-controlled phase of *PIF* gene expression and increased protein stability in the dark (Nozue *et al.*, 2007). Quantification of *PIF4* mRNA during the last part of the night confirmed that there was no difference between *cdf1235* and Col–0; however, partial suppression of the enhanced *PIF4* expression in *gi* mutants was observed in *gi cdf1235* plants (Figure 4a). This result indicates that GI represses growth by suppressing transcription of *PIF4* at the end of the night, and that increased expression of *PIF4* in *gi* mutants is partially due to CDF activity.

Expression of *IAA29*, a direct target of *PIF4*, was measured as an indirect indicator of PIF4 protein activity or abundance (Sun *et al.*, 2013). Under LDs, *IAA29* showed increased mRNA expression in *gi*, correlating with higher *PIF4* mRNA levels (Table S1). Under SDs, the level of *IAA29* mRNA was enhanced in *gi* mutants, particularly at the end of the night (Figure 4b). In *gi cdf1235*, expression of *IAA29* was reduced to levels similar to those in Col–0, indicating that transcription of *IAA29* was sensitive to reduction of *PIF4* expression, and probably reflected decreased accumulation or activity of PIF4 protein (Figure 4b).

The double mutants *gi pif4* and *gi pif5* showed suppressed growth of hypocotyls compared to *gi*', and a triple mutant *gi pif4 pif5* showed a slightly additive effect compared to either double mutant (Figure 4c). Expression of *IAA29* was strongly reduced in *gi pif4* and *gi pif5*, and almost completely abolished in *gi pif4 pif5* (Figure 4d). These results provide genetic evidence that *PIF4* and *PIF5* are required for the enhanced growth of *gi* hypocotyls. Taken together, these data demonstrate the existence of a signaling cascade that, through the GI–CDF module, represses expression of *PIF4* and *IAA29* and ultimately growth.

Mutations in the CDF genes do not affect the length of the circadian period in gi mutant plants

GI is required for proper rhythmicity of the circadian clock, and *gi* mutants show shorter period lengths compared to wild-type (Fowler *et al.*, 1999; Park *et al.*, 1999; Mizoguchi *et al.*, 2005). Cold-responsive genes (Fowler *et al.*, 2005;

Table 1 Freezing tolerance assays

Experiment	Conditions	Genotypes	Number of plants	Number of surviving plants (%)	*P* value
1	Non-acclimated: 10-day-old seedlings exposed to −4°C from ZT9 to ZT15 without any prior cold acclimation	Col–0	201	195 (97.01)	
		gi-100	175	175 (100.00)	0.021
		cdf1235	127	123 (96.85)	0.932
		gi cdf1235	193	185 (95.85)	0.654
2	Non-acclimated: 10-day-old seedlings exposed to −5.5°C from ZT9 to ZT15 without any prior cold acclimation	Col–0	108	50 (46.29)	
		gi–100	88	52 (59.09)	0.074
		cdf1235	129	54 (41.86)	0.493
		gi cdf1235	115	53 (46.08)	0.612
3	Acclimated: 8-day-old seedlings, kept at 4°C for 2 days, 0°C for 6 h, −5°C for 6 h (between ZT9 and ZT15), 0°C for 3 h, 4°C for one night, and then moved to 22°C	Col–0	182	97 (53.29)	
		gi–100	202	151 (74.75)	0.0000
		cdf1235	111	73 (65.76)	1
		gi cdf1235	140	95 (67.85)	0.035
					0.017
4	Acclimated: 14-day-old seedlings, exposed to 4°C for 64 h, 0°C for 5 h, −8°C for 4 h, 0°C for 5 h, 4°C for 20 h, and then moved to 22°C	Col–0	92	59 (64.13)	
		gi–100	89	83 (93.25)	0.0000
		cdf1235	54	35 (64.81)	01
		gi cdf1235	69	55 (79.71)	0.933
					0.103
5	Non-acclimated: 14-day-old seedlings, exposed to 0°C for 5 h, −8°C for 4 h, 0°C for 5 h, 4°C for 20 h, and then moved to 22°C	Col–0	50	7 (14.00)	
		gi–100	72	33 (45.83)	0.0002
		cdf1235	63	7 (11.11)	0.643
		gi cdf1235	72	12 (16.66)	0.667

Seedlings grown under LDs were used. When scoring 14-day-old or cold-acclimated seedlings, the number of plants that showed no sign of damaged was scored. The *P* value was calculated based on the χ^2 distribution, comparing each genotype with Col–0 and according to the null hypothesis that dying, or being damaged by cold, is independent of genotype.

Lee and Thomashow, 2012), *PIF4*, *PIF5* (Nozue *et al.*, 2007) and components of the oscillator, such as *PRR5* and *PRR7* (Figure S1), depend on a functional circadian clock for their wild-type expression patterns. Therefore, the altered cold responses and growth observed in *gi* mutants may be indirectly caused by disturbed clock function, and the CDFs may correct such phenotypes by restoring proper circadian parameters. To test this idea, circadian period length was measured in various genotypes using cotyledon movement assays. In *gi* mutants, shorter period lengths were observed than in wild-type plants (Figure 5a). However, *gi cdf1235* seedlings showed the same period length as *gi* mutants. These data indicate that mutations in the *CDF* genes have no detectable effect on clock periodicity.

A major function of GI is to stabilize the F–box proteins FKF1, ZTL and LKP2 to degrade CDF proteins, promoting *CO* and *FT* transcription and flowering (Kim *et al.*, 2007; Sawa *et al.*, 2007; Fornara *et al.*, 2009). However, *FKF1*, *ZTL* and *LKP2* are also genetically redundant in controlling proper progression and robustness of the circadian clock, as indicated by the arrhythmic features of *fkf1 ztl lkp2* triple mutants (Baudry *et al.*, 2010). GI directly interacts with FKF1 and ZTL proteins to stabilize them and promote their accumulation (Kim *et al.*, 2007; Fornara *et al.*, 2009). The effect of *gi* mutations on circadian parameters is not as severe as that caused by *fkf1 ztl lkp2*, indicating residual FKF1 ZTL LKP2 activity in *gi* mutants or partly independent modes of action on the clock. To investigate how GI and these three F–box proteins interact to regulate the clock and growth, which is a clock-regulated process, we generated a *gi fkf1 ztl lkp2* quadruple mutant, and measured the period length of seedlings grown under continuous light. The *gi fkf1 ztl lkp2* mutants were arrhythmic, with a similar phenotype to *fkf1 ztl lkp2* mutants (Figure 5b), indicating that the mutations in the genes encoding these F–box proteins are genetically epistatic to *GI* in determining period length. Additionally, these plants showed reduced hypocotyl growth under SDs compared to *gi* or Col–0 (Figure 5c), and showed strongly suppressed *PIF4* expression at the end of the night (Figure 5d). These phenotypes cannot be explained by the role of GI as a stabilizer of FKF1 ZTL LKP2, and suggest opposing and independent modes of action for GI and the F–box proteins on growth and *PIF4* gene expression. The *fkf1 ztl lkp2* mutations probably override the effects of the *gi* mutation and light signals on hypocotyl length and gene expression by impairing clock function.

DISCUSSION

GI is central to several signaling pathways, but its molecular function has been most thoroughly described in the context of the photoperiodic flowering pathway. The CDFs act downstream of GI to repress *CO* and *FT* transcription, and here we have shown how the GI–CDF module also

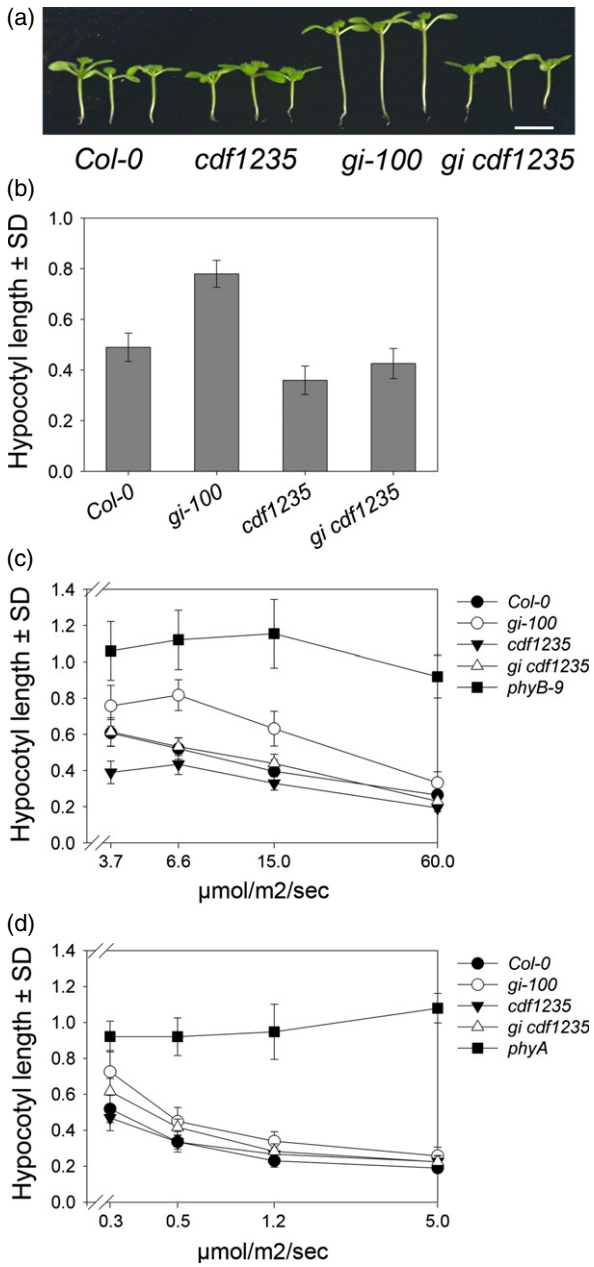

Figure 3. Hypocotyl elongation of *gi* is suppressed by mutations in *CDF* genes.
(a) Seedlings of Col–0, *cdf1235*, *gi* and *gi cdf1235* grown on soil under SD conditions. The photograph was taken after 12 days of growth. Three plants per genotype are shown. Scale bar = 0.5 cm.
(b) Quantification of the phenotypes represented in (a). Seedlings were grown on soil under SD conditions for 10 days. Error bars indicate standard deviation.
(c,d) Fluence response curves of the same genotypes as in (a) under continuous red light (c) or far-red light (d). Mean hypocotyl length is expressed in cm. *phyB–9* and *phyA* are insensitive to growth inhibition by red and far-red light, respectively, and are included as controls. The hypocotyls of at least 20 seedlings were measured. Error bars indicate standard deviation.

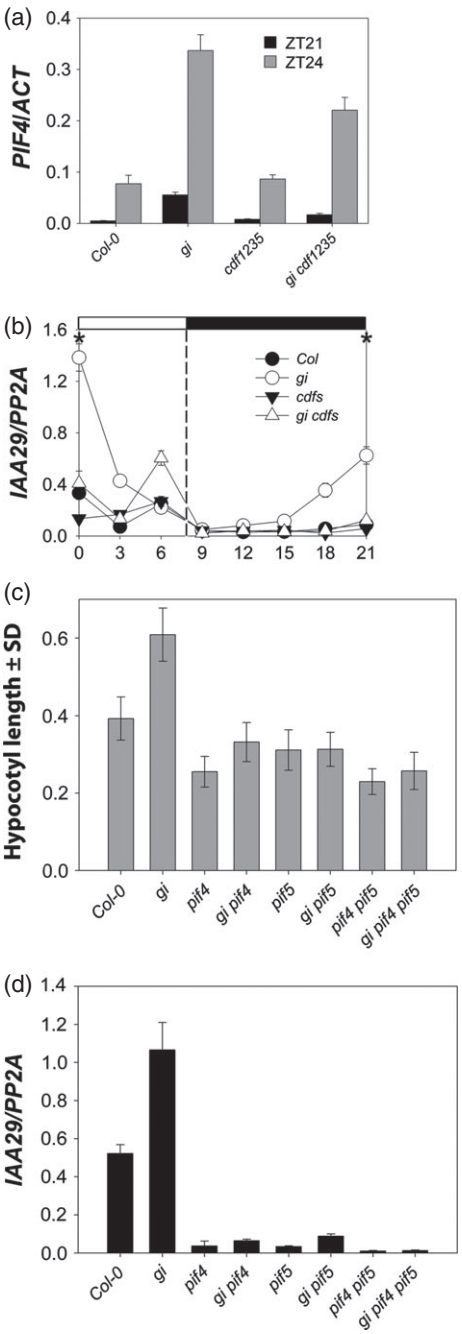

Figure 4. *PIF4* and *PIF5* are required to promote growth of *gi* mutants.
(a) Expression of *PIF4* in the indicated mutants at the end of the night in plants grown under SD conditions. Samples were collected at ZT21 and ZT24.
(b) Diurnal expression of *IAA29* in 10-day-old seedlings of the indicated genotypes grown under SD conditions. The white and black bars on the top of the graph indicate day and night periods, respectively. Asterisks indicate *P* values ≤0.05 determined as described in Figure 2(d).
(c) Quantification of hypocotyl length of the indicated genotypes expressed in cm; seedlings were grown under SD conditions, and 20 seedlings per genotype were measured.
(d) Expression of *IAA29* in the indicated genotypes grown under SD conditions and harvested at ZT1.
Error bars in (a), (b) and (d) indicate standard deviation of three technical replicates.

contributes to processes apparently unrelated to photoperiodic flowering. Cold tolerance and control of hypocotyl growth represent two such processes whose regulation

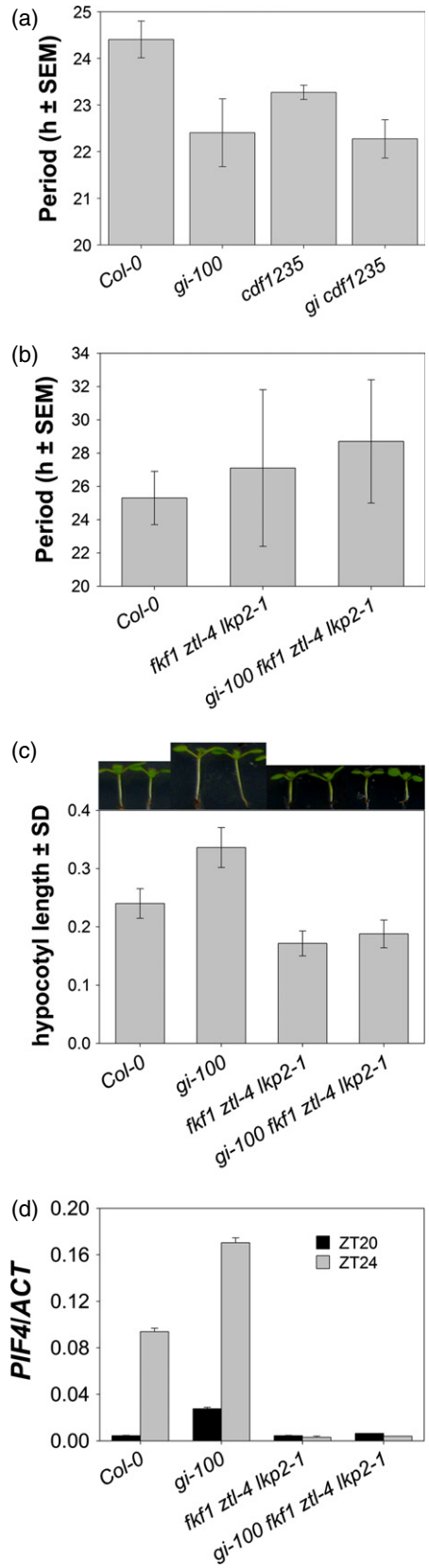

Figure 5. Epistatic interactions between *gi* and *fkf1 ztl lkp2*.
(a,b) Period length measurement in Col–0, *gi*, *cdf1235* and *gi cdf1235* mutants (a), and *fkf1 ztl–4 lkp2–1* and *gi fkf1 ztl-4 lkp2–1* mutants (b). Error bars indicate standard error.
(c) Hypocotyl length of *fkf1 ztl–4 lkp2–1* and *gi fkf1 ztl–4 lkp2–1* arrhythmic mutants compared to Col–0 and *gi*. Error bars indicate standard deviation. Images of two representative seedlings per genotype are shown at the top of each bar.
(d) *PIF4* mRNA expression in the same genotypes as in (c) at ZT20 and ZT24.

involves this module. Depending on the genes targeted by the module, GI or the CDFs act as promoters or repressors of gene expression, showing a high degree of plasticity, and demonstrating that this molecular network motif controls diverse responses to environmental signals.

The GI–CDF regulatory module controls freezing tolerance

In *gi* mutants, mRNAs of *COR* genes were present at higher levels compared to Col–0, and this was probably responsible for conferring the observed increased cold tolerance to acclimated and non-acclimated seedlings. The *cdf1235* mutations suppressed this increase and restored normal freezing tolerance to *gi cdf1235* quintuple mutants. Cold tolerance levels correlated with resistance to reactive oxygen species, which are produced upon freezing and contribute to membrane damage (Baek and Skinner, 2012).

The *cdf1235* mutant showed reduced expression of cold-regulated genes and higher sensitivity to oxidative stress, but did not show increased susceptibility to cold stress, and the effect of mutations in *CDF* genes became obvious only in the background of *gi* mutants. These data indicate that CDFs are not necessary to confer protection from cold stress in wild-type plants, perhaps because other cold stress regulons compensate for the mutations in the *CDF* genes when present in a wild-type background (Nakashima *et al.*, 2009). However, in the *gi* mutant background, increased stability and accumulation of CDF proteins led to increased protection against cold stress. This finding is also supported by recent work in tomato (*Solanum lycopersicum*), in which homologs of the *CDF* genes have been isolated (Corrales *et al.*, 2014). Two such genes, *SlCDF1* and *SlCDF3*, conferred increased resistance to drought and salt stress when ectopically expressed in Arabidopsis. The transcripts of several stress-responsive genes, including *COR15a*, *RD29A* and *ERD10*, and some protective metabolites, including proline, glutamine and sucrose, were more abundant in the plants over-expressing *SlCDF1* and *SlCDF3*, correlating with protection from water deprivation (Corrales *et al.*, 2014).

Overall, these data indicate that the *cdf* mutations are epistatic to *gi* and act antagonistically, similar to what was observed for *CO* transcription. Therefore, the GI–CDF module represents an additional layer of transcriptional regulation of the cold response pathway that is under

photoperiodic control and may mediate its seasonal expression. Our model disagrees with that proposed by Cao *et al.* (2005) suggesting that GI acts in a CBF-independent pathway to promote freezing tolerance. The use of *gi* mutant alleles in a different ecotype with different assay conditions may partly explain such discrepancies.

Exposure to cold stress of Arabidopsis seedlings grown under long photoperiods induces mRNA expression of *GI* itself (Fowler and Thomashow, 2002; Cao *et al.*, 2005). Our experiments have shown that loss of *GI* function enhances freezing tolerance, but it remains to be determined whether an increase in *GI* expression effectively alters cold stress responses. If this were the case, *GI* may antagonize the effect of cold treatments applied under LDs by repressing the expression of genes involved in cold tolerance.

How *COR* genes are regulated by GI and the CDFs is currently unclear, but our data suggest that regulation of *CBF* genes may be the initial step in the signaling cascade mediated by the module. In *gi* and *cdf1235* mutants, *CBF3* is up- and down-regulated, respectively, similar to what occurs for all *COR* genes identified as targets of the module (Data S1). *CBF3* ectopic expression in Arabidopsis results in increased levels of metabolic products of the cold acclimation pathway, including COR15a protein, sugars and proline, and confers higher cold tolerance to non-acclimated seedlings (Gilmour *et al.*, 2000). By controlling expression of a subset of master regulators, including CBF3 and perhaps other CBFs, the GI–CDF module may tune expression of a larger set of downstream effectors. Whether such regulation is mediated by direct binding of CDF proteins to *CBF* loci remains to be tested.

The influence of GI on circadian rhythms may indirectly affect *COR* gene expression, because transcription of the *CBF* and *COR* genes is gated by the circadian clock, starting to increase in the morning and reaching a peak around mid-day (Fowler *et al.*, 2005; Franklin and Whitelam, 2007; Nakamichi *et al.*, 2009; Dong *et al.*, 2011). Circadian regulation of cold responses requires *CCA1* and *LHY*, whose protein products promote *CBF* gene expression by directly binding to the *CBF* loci. Consistent with this observation, in a *cca1 lhy* double mutant, rhythmic expression of the *CBF* genes is abolished, leading to impaired cold responses (Dong *et al.*, 2011). In a *gi* mutant background, the amplitude of expression of *LHY* and *CCA1* is reduced, and this transcriptional change is not consistent with a linear genetic cascade regulating *CBF* genes downstream of *GI*, *LHY* and *CCA1*, because mutations in *GI* result in increased, rather than decreased, *CBF* expression (Mizoguchi *et al.*, 2005; Dong *et al.*, 2011). These data may indicate separate effects of GI on the clock and *CBF* expression. On one hand, GI is necessary for correct circadian clock function, which in turn gates induction of *CBF* genes and leads to production of cryoprotectants at specific times of day. Additionally, GI, through the CDFs, modulates the amplitude of expression of *COR* genes without affecting diurnal cycling of their mRNA. As mutations in the *CDF* genes did not influence period length under constant conditions, CDFs probably represent a more direct regulatory layer modulating the amplitude of *COR* gene expression but not their circadian regulation. Such an arrangement is analogous to the role of GI and the CDFs in regulation of the clock-controlled flowering-time genes *CO* and *FT*.

Distinct roles of the GI–CDF module and F–box proteins on growth

When *gi* mutants were exposed to SDs, hypocotyl growth was enhanced. Mutations in *cdf1235* suppressed growth, restoring normal hypocotyl length. As CDF proteins are more stable in *gi* mutants and accumulate at higher levels compared to wild- type plants, these data indicate that the CDFs promote hypocotyl elongation, at least when over-expressed. Enhanced hypocotyl growth of *gi* mutants also depended on higher expression of *PIF4* and *PIF5* mRNA, and strong suppression of growth was observed in *gi pif4 pif5* mutant seedlings. Therefore, reduction of CDF protein abundance and *PIF4/5* transcription by GI is necessary for proper hypocotyl elongation. We searched for a connection between the two processes by examining the effect of *cdf* mutations on *PIF4* transcription. *PIF4* mRNA levels were reduced in *gi cdf1235* compared to *gi* mutants, correlating with reduced hypocotyl length. However, in *gi cdf1235* mutants, *PIF4* mRNA abundance was still higher than in *cdf1235* plants, despite the fact that hypocotyl length was comparable between *gi cdf1235* and *cdf1235*. Therefore, the phenotypic effect on growth did not fully correlate with *PIF4* mRNA levels. This indicates that higher levels of CDF proteins in the *gi* mutant only partly account for increased transcription of *PIF4*, and suggests that GI controls this process by also affecting other pathways. This conclusion was further strengthened by analysis of the triple *fkf1 lkp2 ztl* mutant, which displayed strongly reduced growth and *PIF4* transcription, yet expressed at least one CDF protein at very high levels (Fornara *et al.*, 2009). This apparent inconsistency may be explained by the *fkf1 lkp2 ztl* mutant being arrhythmic, such that it fails to gate *PIF4* induction at the end of the night, suppressing hypocotyl elongation. Thus, its circadian defect prevails over the molecular effects caused by stabilization of CDF proteins.

FKF1, LKP2 and ZTL act antagonistically to GI when controlling growth, as opposed to their role in control of flowering time, where they act synergistically with GI to reduce CDF protein abundance. Furthermore, the effect of GI on FKF1, LKP2 and ZTL protein stability does not explain the hypocotyl growth defects of the *gi* mutant. These results highlight the need to consider the separate effects of GI on distinct molecular pathways when trying to understand its

function. GI has a profound impact on plant development and on the interaction with environmental cues, in addition to day length. Deciphering the molecular basis underlying this pleiotropy is essential to provide a framework explaining the diverse traits controlled by this protein.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The Col–0 ecotype was used as the wild-type and was the genetic background for all mutants described in this study. The *fkf1–2*, *gi–100*, *cdf1–R cdf2–1 cdf3–1 cdf5–1*, *gi–100 cdf1–R cdf2–1 cdf3–1 cdf5–1*, *fkf1 ztl–4 lkp2–1*, *pif4–2* and *pil6–1* (*pif5*) mutants have been described previously (Huq *et al.*, 2000; Fujimori *et al.*, 2004; Imaizumi *et al.*, 2005; Leivar *et al.*, 2008; Fornara *et al.*, 2009). The *gi fkf1 ztl lkp2* quadruple mutant was obtained by crossing *gi–100* with *fkf1 ztl–4 lkp2–1*. Plants were grown as described previously (Fornara *et al.*, 2009).

Microarray hybridization

Seedlings of Col–0, *cdf1235*, *gi* and *gi cdf1235* were grown for 10 days under LD conditions (16 h light/8 h dark), and three biological replicates were harvested at 12 h after lights on. RNA was prepared using RNeasy columns (Qiagen, www.qiagen.com), and DNA was removed using DNA-*free* reagents (Ambion, http://www.lifetechnologies.com/it/en/home/brands/ambion.html) according to the manufacturer's instructions. The quality of the isolated RNA was assessed using a Nanochip on a Bioanalyzer 2100 system (Agilent, www.agilent.com). Total RNA samples (150 ng) were reverse-transcribed into double-stranded cDNA, and then *in vitro* transcribed in the presence of biotin-labeled nucleotides using a GeneChip 3′ IVT Express kit (Affymetrix, www.affymetrix.com), including poly(A) controls as recommended by the manufacturer. The quality and quantity of the biotinylated cRNA were determined using a NanoDrop ND 1000 spectrophotometer and the Bioanalyzer 2100 system (Nanodrop, www.nanodrop.com). Biotin-labeled cRNA samples (15 μg) were randomly fragmented for 35 min at 94°C in fragmentation buffer (Affymetrix).

Biotinylated cRNA samples were mixed in 250 μl hybridization mix (Affymetrix) containing hybridization controls and control oligonucleotide B2 (Affymetrix). Samples were hybridized onto Affymetrix AGRONOMICS1 Arabidopsis tiling arrays for 16 h at 45°C. Arrays were then washed using an Affymetrix Fluidics Station 450 using the FS450_0004 protocol. An Affymetrix GeneChip Scanner 3000 was used to measure the fluorescence intensity emitted by the labeled targets. Raw array data have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE59996.

Analysis of microarray data

Data were normalized using Robust Multi-array Average, and probesets were defined using the *agronomics1attairgcdf*, resulting in the expression level of 30 014 genes. Differentially expressed genes were determined using LIMMA (Smyth, 2005), based on a linear model with genotype (Col–0, *cdf1235*, *gi* and *gi cdf1235*) and flowering time (normal, early, late and normal, respectively) as factors. Pairwise comparisons were made between Col–0 and each genotype and between *cdf1235* and *gi*. Moreover, a contrast was designed to specifically identify genes that are oppositely regulated in early-flowering (*cdf1235*) versus late-flowering (*gi*) genotypes compared to genotypes whose flowering is similar to Col–0 (*gi cdf1235*). This contrast indicates whether the fold changes late/normal and normal/early are similar to each other but different from 1 (Data S1). The resulting *P* values were corrected for multiple hypothesis testing using the Benjamini–Hochberg procedure.

Gene annotation and gene ontology (GO) clustering were performed using MADIBA (http://madiba.bi.up.ac.za/) and AMIGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi). Phase enrichment was determined using Phaser (http://phaser.mocklerlab.org/).

Quantification of mRNA expression

RNA was isolated from whole seedlings using an RNeasy extraction kit (Qiagen). Residual DNA was removed using a DNA-*free* kit (Ambion). cDNA was synthesized using 3–5 μg total RNA with a Superscript II kit (Invitrogen, http://www.lifetechnologies.com/it/en/home/brands/invitrogen.html) and oligo(dT) primer, according to the manufacturer's instructions. cDNA was diluted to 180 μl with water, and 3 μl were used for subsequent quantitative RT–PCR reactions. Amplified products were quantified using SYBR Green II on a LightCycler 480 system (Roche, www.roche.com). Reactions were performed in triplicate. Primer pairs used for expression analyses are listed in Table S5.

Cotyledon movement assays

The methods for period measurement on the basis of cotyledon movement have been previously described (Hanano *et al.*, 2008). Briefly, seedlings were entrained for 9 days to 12 h/12 h light/dark cycles. Single seedlings were transferred to 1.5% agar blocks containing 3% sucrose, and placed into 25-well square tissue culture dishes maintained in a vertical position. Twenty seedlings for each genotype were transferred according to a randomized block design. The seedlings were entrained as described above for another day, and then placed into a growth chamber for imaging over 1 week under constant white light (25–40 μmol m^{-2} sec^{-1}) at 22°C. Images were obtained every 30 min, and analyzed using Metamorph (http://www.moleculardevices.com) and BRASS (http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm). Period lengths were calculated from leaf movement data by the fast Fourier transform non-linear least-squares method. Mean and SEM values were calculated based on 10–15 leaf traces for each genotype. Three independent experiments gave identical results.

Measurement of hypocotyl length

To construct fluence response curves, seedlings were sterilized and plated on MS medium without sucrose, and stratified for 3 days at 4°C. Plates were kept under constant white light for 6 h, the moved to darkness for 18 h and into E30-LED cabinets (Percival, http://www.percival-scientific.com/) for 3 days under continuous red light. Control plates were wrapped in aluminum foil and maintained under continuous darkness. After 3 days, seedlings were placed flat on agar plates, and hypocotyls were measured using ImageJ (http://rsbweb.nih.gov/ij/). The mean hypocotyl length of each genotype was normalized to that of hypocotyls of seedlings grown in the dark. At least 20 hypocotyls were measured for each light intensity and genotype.

For the SD experiments, seeds were stratified on soil for 3 days at 4°C, and transferred to controlled environments rooms under SD conditions (8 h light/16 h dark). After 10 days, seedlings were harvested and hypocotyls were measured using ImageJ as described above.

Oxidative and cold stress assays

Seedlings were stratified on soil for 3 days at 4°C, and then moved to a growth chamber under LD conditions (16 h light at 22°C/8 h dark at 18°C). Seedlings were then exposed to low temperatures with or without cold acclimation, as indicated in Table 1. Finally, trays were returned to LD conditions, and the percentage of surviving or undamaged seedlings (i.e. seedlings in which no sign of damage was observed on any leaf) was determined a few days after the treatments. *P* values were calculated based on the χ^2 distribution, assuming no correlation between genotypes and damage by cold treatment. Oxidative stress assays were performed as described by Sanchez-Villarreal *et al.* (2013). Two biological replicates were performed and gave similar results.

ACKNOWLEDGEMENTS

The laboratory of G.C. is supported by a core grant from the Max Planck Society. F.F. is supported by European Research Council Starting Grant #260963.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Diurnal expression of genes regulated by GI and/or the CDFs.

Figure S2. Phase of genes differentially expressed in *gi*, *cdf1235* and *gi cdf1235* mutants versus Col–0.

Table S1. Annotation of genes up-regulated or down-regulated in *gi–100* compared to Col–0.

Table S2. Annotation of genes up-regulated or down-regulated in *cdf1235* compared to Col–0.

Table S3. Meta-analysis of genes differentially regulated in *gi–100*, *cdf1235* and *gi cdf1235* mutants, and in publicly available datasets of cycling, cold-, drought- and abscisic acid-regulated genes.

Table S4. Annotation of genes differentially expressed in *gi–100* or *cdf1235* mutants, but not in the *gi cdf1235* quintuple mutant.

Table S5. Primers used in this study for quantification of mRNA expression.

Data S1. Array data from *gi–100*, *cdf1235*, *gi cdf1235* and Col–0.

REFERENCES

Baek, K.H. and Skinner, D.Z. (2012) Production of reactive oxygen species by freezing stress and the protective roles of antioxidant enzymes in plants. *J. Agric. Chem. Environ.* **1**, 34–40.

Baudry, A., Ito, S., Song, Y.H. *et al.* (2010) F-box proteins FKF1 and LKP2 act in concert with ZEITLUPE to control Arabidopsis clock progression. *Plant Cell*, **22**, 606–622.

Cao, S., Ye, M. and Jiang, S. (2005) Involvement of GIGANTEA gene in the regulation of the cold stress response in Arabidopsis. *Plant Cell Rep.* **24**, 683–690.

Corrales, A.R., Nebauer, S.G., Carrillo, L. *et al.* (2014) Characterization of tomato Cycling Dof Factors reveals conserved and new functions in the control of flowering time and abiotic stress responses. *J. Exp. Bot.* **65**, 995–1012.

Covington, M.F., Maloof, J.N., Straume, M., Kay, S.A. and Harmer, S.L. (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biol.* **9**, R130.

Dalchau, N., Baek, S.J., Briggs, H.M. *et al.* (2011) The circadian oscillator gene GIGANTEA mediates a long-term response of the *Arabidopsis thaliana* circadian clock to sucrose. *Proc. Natl Acad. Sci. USA*, **108**, 5104–5109.

David, K.M., Armbruster, U., Tama, N. and Putterill, J. (2006) Arabidopsis GIGANTEA protein is post-transcriptionally regulated by light and dark. *FEBS Lett.* **580**, 1193–1197.

Deng, X., Xu, Y. and Wang, C. (2008) Improving the power for detecting overlapping genes from multiple DNA microarray-derived gene lists. *BMC Bioinformatics*, **9**, S14.

Dong, M.A., Farre, E.M. and Thomashow, M.F. (2011) Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **108**, 7241–7246.

Faure, S., Turner, A.S., Gruszka, D., Christodoulou, V., Davis, S.J., von Korff, M. and Laurie, D.A. (2012) Mutation at the circadian clock gene EARLY MATURITY 8 adapts domesticated barley (Hordeum vulgare) to short growing seasons. *Proc. Natl Acad. Sci. USA*, **109**, 8328–8333.

Feng, S., Martinez, C., Gusmaroli, G. *et al.* (2008) Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. *Nature*, **451**, 475–479.

Fornara, F., Panigrahi, K.C., Gissot, L., Sauerbrunn, N., Ruhl, M., Jarillo, J.A. and Coupland, G. (2009) Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. *Dev. Cell*, **17**, 75–86.

Fornara, F., deMontaigu, A. and Coupland, G. (2010) SnapShot: control of flowering in Arabidopsis. *Cell*, **141**, 550, 550 e551-552.

Fowler, S. and Thomashow, M.F. (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*, **14**, 1675–1690.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G. and Putterill, J. (1999) GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. *EMBO J.* **18**, 4679–4688.

Fowler, S.G., Cook, D. and Thomashow, M.F. (2005) Low temperature induction of Arabidopsis CBF1, 2, and 3 is gated by the circadian clock. *Plant Physiol.* **137**, 961–968.

Franklin, K.A. and Whitelam, G.C. (2007) Light-quality regulation of freezing tolerance in *Arabidopsis thaliana*. *Nat. Genet.* **39**, 1410–1413.

Fujimori, T., Yamashino, T., Kato, T. and Mizuno, T. (2004) Circadian-controlled basic/helix-loop-helix factor, PIL6, implicated in light-signal transduction in *Arabidopsis thaliana*. *Plant Cell Physiol.* **45**, 1078–1086.

Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M. and Thomashow, M.F. (1998) Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J.* **16**, 433–442.

Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D. and Thomashow, M.F. (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.* **124**, 1854–1865.

Hanano, S., Stracke, R., Jakoby, M., Merkle, T., Domagalska, M.A., Weisshaar, B. and Davis, S.J. (2008) A systematic survey in *Arabidopsis thaliana* of transcription factors that modulate circadian parameters. *BMC Genom.* **9**, 182.

Huq, E. and Quail, P.H. (2002) PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in Arabidopsis. *EMBO J.* **21**, 2441–2450.

Huq, E., Tepperman, J.M. and Quail, P.H. (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **97**, 9789–9794.

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A. and Kay, S.A. (2005) FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. *Science*, **309**, 293–297.

Kavi Kishor, P.B., Hong, Z., Miao, G.H., Hu, C.A.A. and Verma, D.P.S. (1995) Overexpression of Δ1-Pyrroline-5-Carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* **108**, 1387–1394.

Kim, S.Y. and Nam, K.H. (2010) Physiological roles of ERD10 in abiotic stresses and seed germination of Arabidopsis. *Plant Cell Rep.* **29**, 203–209.

Kim, W.Y., Fujiwara, S., Suh, S.S., Kim, J., Kim, Y., Han, L., David, K., Putterill, J., Nam, H.G. and Somers, D.E. (2007) ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. *Nature*, **449**, 356–360.

Kim, Y., Yeom, M., Kim, H., Lim, J., Koo, H.J., Hwang, D., Somers, D. and Nam, H.G. (2012) GIGANTEA and EARLY FLOWERING 4 in Arabidopsis exhibit differential phase-specific genetic influences over a diurnal cycle. *Mol. Plant*, **5**, 678–687.

Kim, W.Y., Ali, Z., Park, H.J. *et al.* (2013a) Release of SOS2 kinase from sequestration with GIGANTEA determines salt tolerance in Arabidopsis. *Nat. Commun.* **4**, 1352.

Kim, Y., Han, S., Yeom, M. *et al.* (2013b) Balanced nucleocytosolic partitioning defines a spatial network to coordinate circadian physiology in plants. *Dev. Cell*, **26**, 73–85.

Koornneef, M., Hanhart, C.J. and van der Veen, J.H. (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **229**, 57–76.

Kurepa, J., Smalle, J., Van Montagu, M. and Inze, D. (1998) Oxidative stress tolerance and longevity in Arabidopsis: the late-flowering mutant gigantea is tolerant to paraquat. *Plant J.* **14**, 759–764.

Lee, C.M. and Thomashow, M.F. (2012) Photoperiodic regulation of the C-repeat binding factor (CBF) cold acclimation pathway and freezing tolerance in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **109**, 15054–15059.

Leivar, P. and Monte, E. (2014) PIFs: systems integrators in plant development. *Plant Cell*, **26**, 56–78.

Leivar, P., Monte, E., Al-Sady, B., Carle, C., Storer, A., Alonso, J.M., Ecker, J.R. and Quail, P.H. (2008) The Arabidopsis phytochrome-interacting factor PIF7, together with PIF3 and PIF4, regulates responses to prolonged red light by modulating phyB levels. *Plant Cell*, **20**, 337–352.

de Lucas, M. and Prat, S. (2014) PIFs get BRright: pHYTOCHROME INTERACTING FACTORs as integrators of light and hormonal signals. *New Phytol.* **202**, 1126–1141.

de Lucas, M., Daviere, J.M., Rodriguez-Falcon, M., Pontin, M., Iglesias-Pedraz, J.M., Lorrain, S., Fankhauser, C., Blazquez, M.A., Titarenko, E. and Prat, S. (2008) A molecular framework for light and gibberellin control of cell elongation. *Nature*, **451**, 480–484.

Maibam, P., Nawkar, G.M., Park, J.H., Sahi, V.P., Lee, S.Y. and Kang, C.H. (2013) The influence of light quality, circadian rhythm, and photoperiod on the CBF-mediated freezing tolerance. *Int. J. Mol. Sci.* **14**, 11527–11543.

Matsui, A., Ishida, J., Morosawa, T. *et al.* (2008) Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol.* **49**, 1135–1149.

Miura, K. and Furumoto, T. (2013) Cold signaling and cold response in plants. *Int. J. Mol. Sci.* **14**, 5312–5337.

Mizoguchi, T., Wright, L., Fujiwara, S. *et al.* (2005) Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. *Plant Cell*, **17**, 2255–2270.

Nakamichi, N., Kusano, M., Fukushima, A., Kita, M., Ito, S., Yamashino, T., Saito, K., Sakakibara, H. and Mizuno, T. (2009) Transcript profiling of an Arabidopsis PSEUDO RESPONSE REGULATOR arrhythmic triple mutant reveals a role for the circadian clock in cold stress response. *Plant Cell Physiol.* **50**, 447–462.

Nakashima, K., Ito, Y. and Yamaguchi-Shinozaki, K. (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. *Plant Physiol.* **149**, 88–95.

Nishizawa, A., Yabuta, Y. and Shigeoka, S. (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol.* **147**, 1251–1263.

Niwa, Y., Yamashino, T. and Mizuno, T. (2009) The circadian clock regulates the photoperiodic response of hypocotyl elongation through a coincidence mechanism in *Arabidopsis thaliana*. *Plant Cell Physiol.* **50**, 838–854.

Nozue, K., Covington, M.F., Duek, P.D., Lorrain, S., Fankhauser, C., Harmer, S.L. and Maloof, J.N. (2007) Rhythmic growth explained by coincidence between internal and external cues. *Nature*, **448**, 358–361.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A. and Nam, H.G. (1999) Control of circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. *Science*, **285**, 1579–1582.

Redei, G.P. (1962) Supervital mutants of Arabidopsis. *Genetics*, **47**, 443–460.

Rehrauer, H., Aquino, C., Gruissem, W. *et al.* (2010) AGRONOMICS1: a new resource for Arabidopsis transcriptome profiling. *Plant Physiol.* **152**, 487–499.

Riboni, M., Galbiati, M., Tonelli, C. and Conti, L. (2013) GIGANTEA enables drought escape response via ABA-dependent activation of the Florigens and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1. *Plant Physiol.* **162**, 1706–1719.

Sanchez-Villarreal, A., Shin, J., Bujdoso, N. *et al.* (2013) TIME FOR COFFEE is an essential component in the maintenance of metabolic homeostasis in *Arabidopsis thaliana*. *Plant J.* **76**, 188–200.

Sawa, M. and Kay, S.A. (2011) GIGANTEA directly activates Flowering Locus T in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **108**, 11698–11703.

Sawa, M., Nusinow, D.A., Kay, S.A. and Imaizumi, T. (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. *Science*, **318**, 261–265.

Smyth, G.K. (2005) *Limma: linear models for microarray data*. New York: Springer.

Somers, D.E., Schultz, T.F., Milnamow, M. and Kay, S.A. (2000) ZEITLUPE encodes a novel clock-associated PAS protein from Arabidopsis. *Cell*, **101**, 319–329.

Song, Y.H., Smith, R.W., To, B.J., Millar, A.J. and Imaizumi, T. (2012) FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. *Science*, **336**, 1045–1049.

Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. (1997) *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl Acad. Sci. USA*, **94**, 1035–1040.

Sun, J., Qi, L., Li, Y., Zhai, Q. and Li, C. (2013) PIF4 and PIF5 transcription factors link blue light and auxin to regulate the phototropic response in Arabidopsis. *Plant Cell*, **25**, 2102–2114.

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *Plant J.* **29**, 417–426.

Thomashow, M.F. (2010) Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiol.* **154**, 571–577.