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

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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, freezing, flowering, 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; Koronseef 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 promot-

ers 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), cdf1-1 R cdf2-1 cdf3-1 cdf5-1 (referred to as cdf1235 below) and gi-100 cdf1-1 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 OVER-EXPRESSIO

N of CONSTANS 1 (SOC1) and FRUITFUL (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
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 (Formara 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](image-url)
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 parquat) or H2O2 (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-Vilarreal 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 Δ¹-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 hypo-responsive 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;
and components of the oscillator, such as LKP2 and FKF1 and ZTL proteins to stabilize them and promote their accumulation (Kim et al., 2010; 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 fkh1 ztl lkp2 quadruple mutant, and measured the period length of seedlings grown under continuous light. The gi fkh1 ztl lkp2 mutants were arrhythmic, with a similar phenotype to fkh1 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 fkh1 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...

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)                    | 0.021    |
|                     |                                                        | gi-100              | 175              | 175 (100.00)                   | 0.532    |
|                     |                                                        | cdf1235             | 127              | 123 (96.65)                    | 0.654    |
|                     |                                                        | gi cdf1235          | 193              | 193 (95.85)                    | 0.694    |
| 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)                     | 0.074    |
|                     |                                                        | gi-100              | 88               | 52 (59.09)                     | 0.493    |
|                     |                                                        | cdf1235             | 129              | 54 (41.86)                     | 0.612    |
|                     |                                                        | 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)                     | 0.0000   |
|                     |                                                        | gi-100              | 202              | 151 (74.75)                    | 1        |
|                     |                                                        | cdf1235             | 111              | 73 (65.76)                     | 0.035    |
|                     |                                                        | gi cdf1235          | 140              | 95 (67.85)                     | 0.107    |
| 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)                     | 0.0000   |
|                     |                                                        | gi-100              | 89               | 83 (93.25)                     | 0.01     |
|                     |                                                        | cdf1235             | 54               | 35 (64.81)                     | 0.933    |
|                     |                                                        | gi cdf1235          | 69               | 55 (79.71)                     | 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)                      | 0.0000   |
|                     |                                                        | gi-100              | 72               | 33 (45.83)                     | 0.643    |
|                     |                                                        | cdf1235             | 63               | 7 (11.11)                      | 0.667    |
|                     |                                                        | gi cdf1235          | 72               | 12 (16.66)                     | 0.667    |

Seeds 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 χ² distribution, comparing each genotype with Col-0 and according to the null hypothesis that dying, or being damaged by cold, is independent of genotype.
contributes to processes apparently unrelated to photoperiodic flowering. Cold tolerance and control of hypocotyl growth represent two such processes whose regulation

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 PIFS 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.
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, *SICDF1* and *SICDF3*, 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 *SICDF1* and *SICDF3*, 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 fkh1–2, gi–100, cdf1–R cdf2–1 cdf3–1 cdf5–1, gi–100 cdf1–R cdf2–1 cdf3–1 cdf5–1, fkh1 ztl–4 lkp2–1, pi4–2 and pi6–1 (pi5) 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 fkh1 ztl lkp2 quadruple mutant was obtained by crossing gi–100 with fkh1 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 a RNeasy columns (Qiagen, www.qiagen.com), and DNA was removed using DNA-free reagents (Ambion, http://www.lifetech.net/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 18 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 data have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE99996.

**Analysis of microarray data**

Data were normalized using Robust Multi-array Average, and probesets were defined using the agronomics1attaigrcdf, 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, and late, 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 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.lifetechologies.com/ti/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://miller.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.html). 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://rsweb.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 damaged seedlings (i.e. seedlings in which no sign of damage was observed on any leaf) was determined a few days after the treatments. μ values were calculated based on the χ² 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.

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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.

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