Disruption of the Arabidopsis Circadian Clock Is Responsible for Extensive Variation in the Cold-Responsive Transcriptome[1[C][W][OA]]

Zuzanna Bieniawska[2,3], Carmen Espinoza[2], Armin Schlereth, Ronan Sulpice, Dirk K. Hincha, and Matthew A. Hannah[4]*

Max-Planck-Institut für Molekulare Pflanzenphysiologie, D–14424 Potsdam, Germany

In plants, low temperature causes massive transcriptional changes, many of which are presumed to be involved in the process of cold acclimation. Given the diversity of developmental and environmental factors between experiments, it is surprising that their influence on the identification of cold-responsive genes is largely unknown. A systematic investigation of genes responding to 1 d of cold treatment revealed that diurnal- and circadian-regulated genes are responsible for the majority of the substantial variation between experiments. This is contrary to the widespread assumption that these effects are eliminated using paired diurnal controls. To identify the molecular basis for this variation, we performed targeted expression analyses of diurnal and circadian time courses in Arabidopsis (*Arabidopsis thaliana*). We show that, after a short initial cold response, in diurnal conditions cold reduces the amplitude of cycles for clock components and dampens or disrupts the cycles of output genes, while in continuous light all cycles become arrhythmic. This means that genes identified as cold-responsive are dependent on the time of day the experiment was performed and that a control at normal temperature will not correct for this effect, as was postulated up to now. Time of day also affects the number and strength of expression changes for a large number of transcription factors, and this likely further contributes to experimental differences. This reveals that interactions between cold and diurnal regulation are major factors in shaping the cold-responsive transcriptome and thus will be an important consideration in future experiments to dissect transcriptional regulatory networks controlling cold acclimation. In addition, our data revealed differential effects of cold on circadian output genes and a unique regulation of an oscillator component, suggesting that cold treatment could also be an important tool to probe circadian and diurnal regulatory mechanisms.

Low temperature is a key signaling cue and a primary determinant of plant growth, development, and survival (Johanson et al., 2000; Bastow et al., 2004). Work to elucidate the molecular mechanisms of plant low-temperature responses has focused on Arabidopsis (*Arabidopsis thaliana*), which, like many important crop plants, is able to cold acclimate, the process by which temperate plants increase their freezing tolerance in response to low but nonfreezing temperatures (Thomashow, 1999). This complex adaptive trait is associated with massive molecular responses involving thousands of transcripts (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002; Hannah et al., 2005) and hundreds of metabolites (Cook et al., 2004; Kaplan et al., 2004). Genetic approaches have defined some of the key regulators of cold acclimation and their regulation of some of the complex molecular responses. The best characterized of these are C-REPEAT BINDING FACTOR1 (CBF1), CBF2, and CBF3 (Gilmour et al., 1998), also known as DEHYDRATION RESPONSIVE ELEMENT BINDING1b (DREB1b), DREB1c, and DREB1a, respectively (Liu et al., 1998). Overexpression of CBFs induces cold-regulated (COR) genes, causes similar metabolic changes as low temperature exposure, and increases freezing tolerance (Gilmour et al., 2000). Recently, the use of natural variation has revealed that CBF2 likely contributes to the different acclimated freezing tolerance of ecotypes Cape Verde Islands and Landsberg *erecta* (Alonso-Blanco et al., 2005), and elevated CBF expression, concomitant with CBF-dependent molecular changes, is associated with high acclimated and nonacclimated freezing tolerance (Hannah et al., 2006). Positive (Chinnusamy et al., 2003) or negative (Lee et al., 2001) regulators of CBF and pathways independent of (Zhu et al., 2004; Xin et al., 2007) or overlapping with (Vogel et al., 2005) the CBF pathway have also been described. The concentration of cytosol-free calcium ($[Ca^{2+}]_{CYT}$) is rapidly increased by exposure to low temperature (Knight et al., 1991), and this correlates

[1] This work was supported by the Max-Planck Society.

[2] These authors contributed equally to the article.

[3] Present address: John Innes Centre, Norwich NR4 7UH, UK.

[4] Present address: Bayer Cropscience N.V., Technologiepark 38, 9000 Gent, Belgium.

* Corresponding author; e-mail matthew.hannah@bayercropscience.com.

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with the induction of the *COR78* gene, also known as RD29A/LTI78 (Henriksson and Trewavas, 2003).

Circadian clocks are the internal molecular chronometers that most organisms use to measure time. These allow the anticipation of, and response to, the environmental changes that accompany the daily rotation of the earth. The clock controls many important processes, is responsible for generating circadian rhythms at both the molecular (Harmer et al., 2000) and the physiological (Webb, 2003) levels, and contributes to plant fitness (Dodd et al., 2005). Circadian rhythms are cycles that persist in continuous environmental conditions with a period of approximately 24 h and are stable over a wide range of physiological temperatures, referred to as temperature compensation (McClung, 2006). These rhythms are entrained by environmental time cues (termed by the German *zeitgeber*) such as light-dark or temperature cycles (McClung, 2006), although their effects on entrainment can be different (Michael et al., 2003a; Boothroyd et al., 2007). The circadian clock in plants is composed of a complex network of interlocking positive and negative feedback loops. The primary feedback loop is composed of the related MYB transcription factors (TFs) CIRCADIAN AND CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which peak near dawn and together regulate the expression of the evening-expressed pseudoresponse regulator (PRR) TIMING OF CAB EXPRESSION1 (TOC1/PRR1; Alabadi et al., 2001). Recent modeling of the circadian oscillator, incorporating experimental data, has added additional components and feedback loops to this core, resulting in a three-loop model (Locke et al., 2005, 2006; Zeilinger et al., 2006). A morning oscillator loop between LHY/CCA1 and PRR7/PRR9, and an evening oscillator loop between TOC1 and an unknown component Y, are linked via the core oscillator loop between LHY/CCA1 and TOC1 involving an unknown component X (Locke et al., 2006; Zeilinger et al., 2006). The circadian-regulated gene *GIGANTEA* (*GI*; Fowler et al., 1999; Sawa et al., 2007) is a strong candidate as a component of the unknown factor Y (Locke et al., 2005, 2006). However, it is clear from recent data on the molecular basis of temperature compensation that further missing elements need to be incorporated. Temperature compensation between 12°C and 27°C involves a critical role for GI, likely via temperature-dependent interaction with CCA1 and LHY (Gould et al., 2006). At 27°C, FLOWERING LOCUS C (FLC) mediates a period lengthening in the accession Cape Verde Islands versus Landsberg *erecta*, and this may involve increased expression of *LUX ARRHYTHMO* (*LUX*; Edwards et al., 2006), which encodes an evening-expressed MYB TF essential for circadian rhythms (Hazen et al., 2005). This period lengthening by *FLC* is consistent with a role in suppressing the induction of flowering by elevated temperatures (Balasubramanian et al., 2006).

Despite our understanding of the circadian oscillator, life in a rotating world is not as simple as the sine waves it generates under constant environmental conditions. Plants grow under daily cycles of light and temperature that integrate with the circadian clock, resulting in complex diurnal molecular and physiological rhythms. A seminal demonstration of this integration was that *CHLOROPHYLL A/B BINDING PROTEIN2* (*CAB2*) expression is induced by a light pulse during the subjective day but not during the night, a phenomenon known as ''gating'' (Millar and Kay, 1996). An opposite example is that while circadian-regulated genes make a major contribution to diurnal expression changes, the expression of many of these genes is also regulated by endogenous sugar levels (Blasing et al., 2005). At the physiological level, the diurnal growth phase is dramatically shifted relative to circadian conditions (Nozue et al., 2007). Hormones, light, and the circadian clock have all been shown to be important factors regulating hypocotyl growth (for review, see Nozue and Maloof, 2006). The integration of the circadian regulation of transcript levels and light regulation of protein abundance for two growth-promoting basic helix-loop-helix TFs was recently shown as a molecular basis for diurnal growth (Nozue et al., 2007). Further complexity is demonstrated by the circadian gating of signaling and growth responses to the plant hormone auxin (Covington and Harmer, 2007).

Therefore, it is reasonable to assume interactions between low temperature and the circadian clock and that understanding the response of plants to low temperature will require consideration of diurnal environmental changes. Indeed, there is evidence that some circadian-regulated genes are cold responsive (Kreps et al., 2002) and that the CBF TFs and their target genes are circadian regulated (Edwards et al., 2006). Furthermore, the circadian clock gates the cold induction of the CBF TFs (Fowler et al., 2005), $[Ca^{2+}]_{CYT}$ signals, and the expression of COR78 (Dodd et al., 2006). It was also proposed that winter causes a disruption of central oscillator components in chestnut (*Castanea sativa*; Ramos et al., 2005) and noted that low temperature may affect oscillator function in Arabidopsis (Gould et al., 2006). A possible involvement of GI in the cold response was proposed based on its cold induction and the reduced constitutive and acclimated freezing tolerance of the *gi-3* mutant (Cao et al., 2005).

Despite this emerging data on the reciprocal interactions between the circadian clock and cold signaling, understanding of how low temperature affects the circadian clock is lacking. Furthermore, whether circadian and diurnal regulation may influence the findings of previous efforts to elucidate cold response pathways is completely unknown. Here, we first use microarray expression data, both from public databases and our own experiments, to quantify the influence of circadian and diurnal regulation on the identification of cold-responsive genes. We then use targeted expression studies by quantitative reverse transcription (qRT)-PCR to demonstrate that these differences are largely due to the fact that under normal diurnal light-dark conditions, cold dampens the cycles of oscillator com-

ponents and disrupts those of some circadian output genes, while in circadian conditions oscillator components also stopped cycling. We further demonstrate time-of-day dependence by showing stronger, more abundant induction of TFs in the morning than in the evening. These data also reveal differential effects of cold on circadian oscillator and output genes, thus providing novel insight into clock function and revealing a unique regulatory mechanism for the clock component *LUX*.

RESULTS

Diversity in the Identification of Cold-Responsive Genes

Given the lack of a common standard for studying the cold responses of plants, it is generally accepted that developmental and environmental influences lead to differences between independent studies. However, the magnitude of these differences and the dominant causes of variation have not been systematically investigated. One obvious source of variation is the thousands of genes that are diurnally regulated. Most studies claim, and it is widely assumed, that diurnal or circadian effects are excluded by harvesting control plants at the same time of day as cold-treated plants or by using plants grown in continuous light. To test this assumption and to determine which factors have the greatest impact on the identification of cold-responsive genes between independent experiments, we assembled a large set of expression data from public databases and from our own experiments (Table I). To limit the number of variables between experiments, all used a cold treatment of approximately 24 h, and control plants were always harvested at the same time of day as cold-treated plants. Other experimental factors, such as growth media, developmental stage, and light intensity and duration, were not standardized and showed considerable variation. With respect to diurnal regulation, three different light regimes were employed. First, plants growing under diurnal conditions were transferred to cold under continuous light. Second, plants were grown under continuous light during growth and cold treatment. Third, control and cold-treated plants were grown under diurnal conditions.

To minimize technical differences, we only considered Affymetrix ATH1 hybridizations and reanalyzed all data using the same procedure resulting in $\log_2$ differences of the cold treatment minus the control. To ensure the detection of experiment-specific responses, any gene that was detected in at least one experiment was retained. Although a generally consistent cold response was indicated by the highly significant correlation between all experiments ($r = 0.47$–0.81, Pearson correlation, $P < 2.2 \times 10^{-308}$ [minimum float in R]; Supplemental Table S1), this concealed massive underlying differences. As a simple measure of these differences, we counted the number of genes that were more than 2-fold changed in one experiment but were changed less than 2-fold in the other. The average pair-wise difference between experiments was around 50%, with a maximum of 71%, and often amounted to more than 3,000 genes (Supplemental Table S2). Given such large differences, it is important to understand which factors are mainly responsible.

To identify the factors responsible for this diversity (in the statistical sense of variance), we performed principal component analysis (PCA; Fig. 1). PCA is an unsupervised method to separate samples based on the underlying coherent variation between them. The contribution of each gene to the separation by a given principal component (PC) is shown by its value in the ''loadings'' for that PC. Comparisons of the loadings for the first five PCs, together explaining more than 70% of the total variance between experiments, revealed a highly significant overlap ($P = 7 \times 10^{-41}$ to 1×10^{-122}, Fisher's exact test) with diurnally regulated transcripts (Table II). As circadian and sugar regulation make the most significant contributions to the diurnal regulation of gene expression (Blasing et al., 2005), we also considered overlap with diagnostic sets of circadian-regulated (Edwards et al., 2006) and sugar-regulated (Solfanelli et al., 2005) genes. The highest overlap with diurnal-regulated genes was observed for PC 2, 4, and 1, respectively, while for circadian-regulated genes the order was reversed (i.e. PC 1, 4, and 2; Table II; Supplemental Fig. S2). More detailed comparisons revealed that transcripts contributing most to the positive and negative loadings for PC 1, 2, and 4 clearly peaked at different times of the day (phase) during a circadian time course (Fig. 2). The overlap for PC 3 and 5 was lower and showed less coordinated time-of-day regulation. To explain the higher diurnal, but lower circadian, contribution to PC 2, we reasoned that sugar signaling may be involved. Comparison of the loadings with Suc-regulated genes (Solfanelli et al., 2005) showed a striking overlap for just PC 2 (Table II; Supplemental Fig. S2), and more detailed comparison clearly indicated the separate contributions of Suc up- and down-regulated transcripts (Supplemental Fig. S1).

Comparison of the experimental factors (Table I) allowed us to determine the most likely basis for each PC. The time-of-day effect underlying PC 1 was most likely an additive effect of the type and timing of the cold treatment. Experiments A and B used cold treatment in continuous light, and experiments A and i started the cold treatment shortly (2–3 h) after dawn; the most extreme experiment (A) shared both factors. Similarly, time-of-day factors were also most likely to contribute to the fourth PC, as the two most extreme experiments (h and k) both used a cold treatment that started in the middle of the light period (Table II). The diurnal regulation of genes contributing to PC 2 was more likely related to their regulation by sugar than by their circadian regulation alone (Table II; Supplemental Fig. S2). PC 2 mainly separated experiment C, but the described experimental conditions did not easily explain this. PC 3 and 5, which had lower overlap with

Table I. *Cold treatment expression profiling data sets used in this study*

Experimental details are shown for the 11 data sets used to investigate the main contributions to variation in the identity of cold-responsive genes. Labeling is as in Figure 1. Experiments are denoted by letters, with lowercase indicating soil-grown plants. Bold, italic, and underlined typefaces indicate the light regime: bold, continuous light for control and cold; italic, diurnal for control and continuous light for cold; underlined, diurnal for control and cold. The Light columns show both intensity (μmol m^{-2} s^{-1}) and duration. The Age column gives the age in days (d) or, where available, the growth stage (Boyes et al., 2001). MS, Murashige and Skoog; NA, not applicable.

Identifier	Control Plants		Cold Treatment						Tissue	Reference
	Medium	Temperature	Light	Age	Temperature	Time	Light	Start		
A	MS Liquid	24°C	150 *16 h*	18 d	3°C	24 h	60 *24 h*	ZT3	Shoot	Kilian et al. (2007)
B	MS Agar, 3% Suc	22°C	NA *16 h*	14 d	0°C	24 h	NA *24 h*	ZT12	Seedling	Lee et al. (2005)
C	MS Agar	21°C	100 **24 h**	1.1 4	4°C	24 h	100 **24 h**	NA	Shoot	Nottingham Arabidopsis Stock Centre NASCARRAYS-70
D	B5 Agar	24°C	100 **24 h**	10 d	4°C	24 h	25 **24 h**	NA	Seedling	Vogel et al. (2005)
e	Soil	22°C	100 **24 h**	18 d	4°C	24 h	25 **24 h**	NA	Shoot	Vogel et al. (2005)
f	Soil	20°C 18°C	150 16 h	3.70	4°C	22 h	90 16 h	ZT14	Shoot	New
g	Soil	20°C 18°C	150 16 h	3.7 0	4°C	26 h	90 16 h	ZT14	Shoot	New
h	Soil	20°C 18°C	150 16 h	3.90	4°C	24 h	90 16 h	ZT8	Leaf discs	New
i	Soil	20°C	125 15 h	21 d	4°C	24 h	125 15 h	ZT2	Shoot	Kaplan et al. (2007)
j	Soil	21°C 16°C	150 16 h	3.70	4°C	22 h	90 16 h	ZT4	Shoot	New
k	Soil	21.5°C	NA 8 h	1.12	4°C	24 h	Same 8 h	ZT4	Shoot	Nottingham Arabidopsis Stock Centre NASCARRAYS-24

diurnally regulated genes, were most likely based on differences in growth media and intraexperiment variation, respectively. For PC 3, there was a clear division between the soil-grown plants and those grown either on plates (B, C, and D) or in hydroponics (A), while PC 5 mostly separated replicates from experiment B.

In summary, there are massive differences in cold-responsive genes between independent studies, and despite the widely held belief that diurnal effects are excluded by the use of paired controls, our meta-analysis revealed that diurnally regulated genes are the dominant source of variation between experiments. This seems to involve both direct time-of-day effects from circadian-regulated genes and indirect contributions from sugar-regulated genes.

The Effect of Low Temperature on the Circadian Clock

Given that the massive diurnal effects on the identification of cold-responsive genes were not previously acknowledged, the underlying mechanism has not been investigated. Investigations with other species showed, for example, winter disruption of oscillator components in chestnut (Ramos et al., 2005) and the cold interruption of clock-regulated transcription in chilling-sensitive tomato (*Solanum lycopersicum*; Martino-Catt and Ort, 1992), indicating that investigating the effects of cold on the clock in Arabidopsis would be informative. However, previous work in Arabidopsis, showing either the effect of cold on three circadian-regulated output genes (Kreps and Simon, 1997) or the possible effect of cold on oscillator components (Gould et al., 2006), is not sufficient to interpret the effects we identified. To resolve the effects of cold on the circadian clock and the genes it controls, and thus interpret the diurnal and circadian effects in our PCA, we performed targeted expression analysis of clock components and output genes using qRT-PCR. This was done using plants transferred to cold either under diurnal conditions or under continuous light. Initial experiments mimicked our previously used 14-d cold treatment at 4°C in 16-h long days and starting in the middle of the day (Rohde et al., 2004; Hannah et al., 2005, 2006). We sampled cold-treated plants every 4 h on days 1, 2, 7, and 14 and control plants on day 1. The most obvious conclusion from these data was that the majority of oscillator components, after an initial cold response, showed diurnal cycles with dramatically reduced amplitude but similar peak expression in the cold as under control conditions (Fig. 3). The initial response (4–20 h) after transfer to cold was often distinct from that observed on days 2, 7, and 14 (Fig. 3), and most oscillator genes were initially induced, or at least expression did not decline as in control plants. Interestingly, reduced expression amplitude was not a universal effect, as *LUX* expression was maintained at the same amplitude under normal and cold conditions, although cold rendered *LUX* expression imme-

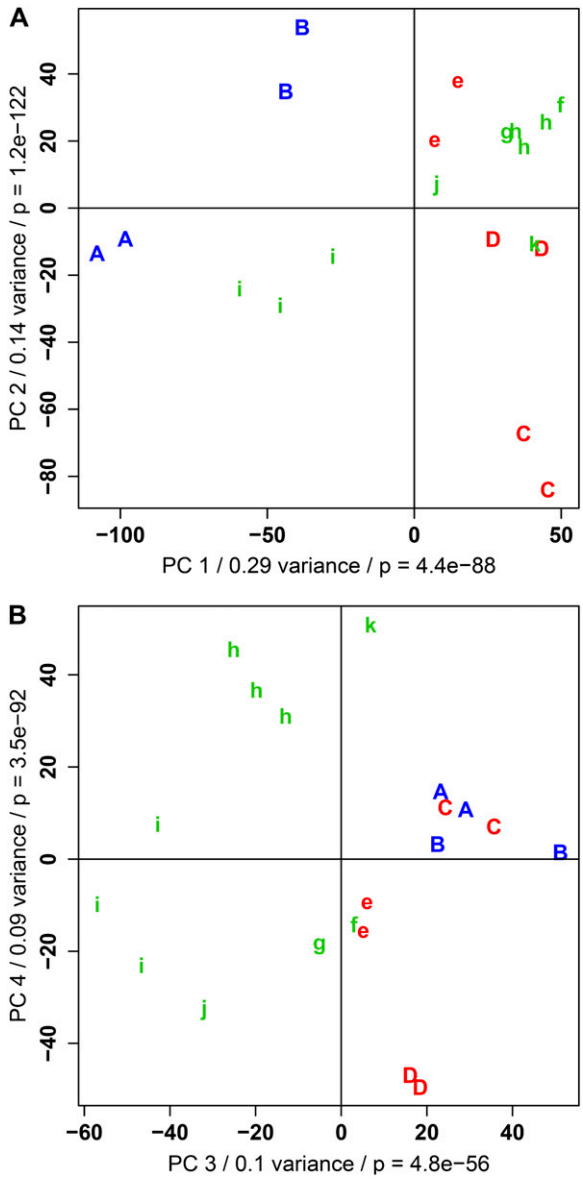

Figure 1. Diurnal regulation makes major contributions to cold-responsive transcriptome differences between experiments. PCA was performed on several independent studies investigating gene expression after 1 d of cold treatment (Table I). GCRMA expression estimates (Wu et al., 2004) were used to calculate the cold minus control $\log_2$ differences. Probe sets that were detected in at least one experiment were retained. Data were mean centered and plotted using classical PCA (Stacklies et al., 2007). Samples from each experiment are denoted by letters, with lowercase denoting soil-grown plants. Colors indicate the light regime: red, continuous light for control and cold; blue, diurnal for control and continuous light for cold; green, diurnal for control and cold. A, PC 1 versus PC 2. B, PC 3 versus PC 4. Axis labels indicate the proportion of the total variance explained by each PC and the *P* value (Fisher's exact test) for the significance of the overlap between the top 500 genes contributing to it and those that are diurnally regulated (Blasing et al., 2005; Table II). [See online article for color version of this figure.]

diately responsive following dawn rather than with the 4-h delay observed under control conditions.

Among circadian-regulated genes, we monitored the expression of four standard circadian output marker genes, *CCR1* and *CCR2*, *CAB2*, and *CATALASE3* (*CAT3*), as well as the cold- and circadian-regulated *CBF* and *COR* genes (Harmer et al., 2000; Edwards et al., 2006). *CBF1* to *CBF3*, *COR78*, *COR47*, and *COR15a* were all circadian and diurnally regulated at normal temperatures under our conditions (Figs. 3 and 4). In contrast to the clear low-amplitude cycles of the core oscillator, no consistent cycles were observed at low temperature for any of the four standard output genes. Significantly, after their initial response, *CBF1* to *CBF3* clearly also cycled at low temperature under light-dark conditions, but similar to the standard clock output genes, the *COR* genes did not (Fig. 3). It was previously shown using northern-blot analysis that under similar cold conditions, *CAB1* and *CCR2* expression was reduced and elevated, respectively (Kreps and Simon, 1997). We found that the expression of *CAB2* and *CCR2* was similar to their maximum expression under diurnal conditions, while *CAT3* was closer to its diurnal minimum (Fig. 3). *CCR1* was initially induced, but on day 2 it declined toward its diurnal minimum, where expression was subsequently maintained. To eliminate the possible effects of the change in light intensity concomitant with our cold treatment, we repeated experiments growing the plants under both normal light (150 μmol m^{-2} s^{-1}) and a light intensity identical to that during the cold treatment (90 μmol m^{-2} s^{-1}). The results were highly similar under both conditions (Fig. 3; Supplemental Fig. S3). We then measured the same genes under continuous light to determine whether circadian function persisted. The experiments were repeated by transferring plants to continuous light at the middle of the day (Supplemental Fig. S4) or 2 h before dusk (Fig. 4). Without exception, the cycling of clock oscillator and output gene mRNA levels appeared to become arrhythmic. Although the expression after 2 d was similar and clearly arrhythmic, the timing of the loss of rhythmic expression was different depending on the time of day the plants were transferred to continuous light (Fig. 4; Supplemental Fig. S4). Arrhythmia seemed to occur more rapidly for most genes when transferred at *zeitgeber* time 8 (ZT8) rather than at ZT14. Generally, the first expression increase that occurred after the transfer to continuous conditions was also partly preserved in the cold. However, once genes reached their circadian maxima, or for genes that were at their maxima at transfer, rhythmic expression was more quickly lost. In other words, it seemed that transcript decline was inhibited; thus, similar to diurnal conditions, most genes clamped to high expression (Fig. 4; Supplemental Fig. S4). Interestingly, this was not the case for all genes, as under diurnal conditions the expression of *CCR1* moved slowly and *CAT3* moved rapidly toward minimum levels.

To summarize, we demonstrate that under diurnal conditions in the cold, clock oscillator components and

some output genes dampened over time to low-amplitude, high-abundance cycles, while standard clock output genes stopped cycling. A unique situation was identified for the clock gene *LUX*, which continued high-amplitude cycles, albeit with advanced phase. In continuous light at 4°C, all genes eventually became arrhythmic, indicating that circadian function was disrupted.

Time-of-Day Dependence of the Cold Response

One aspect of cold-circadian interactions that has been reported previously is the gating of the low-temperature induction of *CBF1* to *CBF3* by the circadian clock (Fowler et al., 2005). However, other studies concluded that the circadian clock did not affect *CBF3* cold induction (Maruyama et al., 2004), and although two other cold-induced TFs, *RAV1* and *ZAT12*, were also gated (Fowler et al., 2005), the wider occurrence of time-of-day effects on TF induction is unknown. To investigate TF gating under diurnal conditions, we measured TF cold induction by qRT-PCR at 2 h after dawn (ZT2) and 2 h before dusk (ZT14) and included diurnal controls before and after cold treatment. This was done using an updated version of a qRT-PCR platform (Czechowski et al., 2004) quantifying the expression of approximately 1,900 TFs (including the *CBF*s). Initial experiments at 0.75, 1.5, 3, and 6 h indicated maximum *CBF* expression at 3 h after cold treatment (data not shown), so this time point was subsequently used. In agreement with Fowler and coworkers (2005), we found that *CBF* induction was gated relative to the initial and paired controls and absolute abundance was greater after morning cold induction under normal light conditions (Table III). When plants grown under low light were transferred to 4°C (i.e. maintaining the same light intensity), the absolute transcript abundance of both *CBF2* and *CBF3* was also gated, but less so than using plants grown under normal light. In contrast, the absolute transcript abundance of *CBF1* was 5-fold higher after cold induction in the evening compared with the morning in the low-light experiment (Table III).

At the global level, we first selected TFs that were changed at least 4-fold relative to both the before-cold and paired controls in two independent experiments (data not shown). We then measured the resulting 69 up-regulated and 14 down-regulated candidates using two independent experiments with five biological replicates each. The two experiments used different light intensities to ensure the identification of robust cold-regulated TFs. Applying stringent criteria (*t* test, $P < 0.05$ and >4-fold change compared with both controls in both experiments), we confirmed 56 up-regulated and four down-regulated genes. The low overlap for repressed genes was predominantly caused by one or two outliers among the five samples pooled in the original screening (data not shown). Among the up-regulated genes, 48 and 27 met our criteria for being up-regulated after cold treatment at ZT2 and ZT14, respectively. These data show that, even using identical treatment conditions, 75% more TFs were identified as cold induced in the morning compared with the evening. The gating of relative cold induction is clearly visible for a large number of TFs (Fig. 5, cold induction). In addition to relative induction, we investigated the gating of absolute cold-induced transcript abundance of these TFs. In common with the numbers of genes, the maximum transcript abundance for the majority (42) of these genes was achieved after cold induction at ZT2 (Fig. 5, Cm–Ce [cold morning to cold evening]). This is mostly due to increased cold induction rather than to differences in the initial transcript abundance. Indeed, where different, initial abundance tended to be higher at ZT14 than at ZT2 (Fig. 5, ZT2–ZT14). As we used diurnal conditions, we considered that the observed gating could be due to light-dependent cold induction (i.e. 3 h of light for the morning cold treatment versus 2 h of light/1 h of dark for the evening). However, an independent experiment investigating morning cold induction in either the light or dark indicated that the influence of such an effect was minimal (M.A. Hannah and L. Willmitzer, unpublished data). Interestingly, many other AP2/EREBP family TFs were also cold induced and gated in the same way as *CBF1* to *CBF3* and *RAV1* (Fig. 5). To quantify this, we performed overrepresentation analysis on these morning-gated TFs (>2-fold absolute gating), which showed that members of the AP2/EREBP and C2C2(Zn) CO-like TF families were significantly overrepresented (Fisher's exact test, $P = 7 \times 10^{-8}$ and $P = 2 \times 10^{-4}$, respectively).

Table II. *Significant overlap between diurnal-, circadian-, and Suc-regulated genes and those contributing to variance between cold experiments*

Following PCA (Fig. 1) to identify the major differences between independent experiments to identify cold-responsive genes, we extracted the top 500 genes contributing to PC 1 to PC 5. These genes were compared with diagnostic sets of diurnal-regulated (Blasing et al., 2005), circadian-regulated (Edwards et al., 2006), and Suc-regulated (Solfanelli et al., 2005) genes, and the significance of the overlap was calculated. Absolute numbers of genes as well as *P* values from Fisher's exact test are shown. The numbers of genes in parentheses indicate the size of each diagnostic gene set. Venn diagrams showing the overlap between gene lists are shown in Supplemental Figure S2.

PC	Diurnal (3,409)		Circadian (3,480)		Suc (1,890)	
PC 1	303	4.4×10^{-88}	255	1.2×10^{-51}	137	1.5×10^{-23}
PC 2	342	1.2×10^{-122}	240	9.4×10^{-43}	263	1.3×10^{-118}
PC 3	259	4.8×10^{-56}	161	2.4×10^{-09}	123	3.1×10^{-17}
PC 4	308	3.5×10^{-92}	245	1.2×10^{-45}	133	9.8×10^{-22}
PC 5	234	7.3×10^{-41}	152	4.3×10^{-07}	120	6.9×10^{-16}

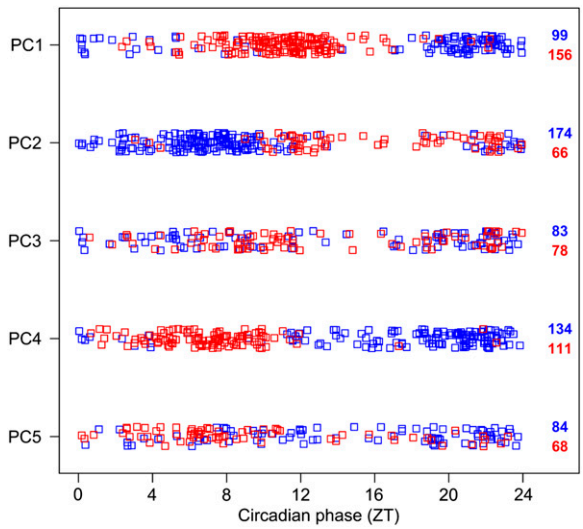

Figure 2. Circadian-regulated genes make coordinated phase-specific contributions to the major differences between experiments. Following PCA (Fig. 1) to identify the main differences between independent experiments to identify cold-responsive genes, we extracted the top 500 genes contributing to PC 1 to PC 5. These genes were separated into those that contributed positively (blue) or negatively (red) to the separation. To visualize the time of day these genes have maximum expression, the numbers and the phases of those genes classified as circadian regulated (Edwards et al., 2006) are plotted for each PC. [See online article for color version of this figure.]

These data confirmed the gating of the *CBF1* to *CBF3* and *RAV1* TFs, and measurements on essentially all Arabidopsis TFs revealed that time of day influenced the cold induction of many TFs, particularly among AP2/EREBP and C2C2(Zn) CO-like family members. Around 75% more TFs were cold responsive in the morning than in the evening, and transcripts often reached higher levels during cold treatment in the morning.

Impact of Circadian and Diurnal Regulation on the Identification of Cold-Responsive Genes

Given these data, we predicted that circadian-regulated genes would have been identified as cold responsive in previous studies and that, as oscillations are dampened or stopped in the cold, genes that peak at different times of day (phase) should show coordinated up- or down-regulation by cold, leading to phase-dependent differences between experiments. This supervised analysis of the circadian phase of cold-responsive genes could also reveal patterns that were not evident in our unsupervised PCA. We performed these analyses using a published circadian time series (Edwards et al., 2006) to identify the expression phase of cold-induced and cold-repressed genes for each experiment. This revealed clear differences in the likelihood of circadian-regulated genes of different phase to be up- or down-regulated by cold (Fig. 6). As predicted, cold up- and down-regulated genes had an opposite relationship (i.e. one overrepresented and the other underrepresented) at many phases in most experiments. Furthermore, the reciprocal regulation of genes at opposite phase (e.g. dawn/dusk) was also often observed.

As suggested by our PCA, there is a clear experiment-specific bias in the phase of cold-responsive genes. Experiments A and B, which used cold treatment in continuous light, have significant overrepresentation of cold up-regulated genes among those with phases ZT10 and ZT12, while those of ZT0 to ZT6 were significantly down-regulated (Fig. 6). A closely related pattern was shown by experiment i, which grouped together with these experiments in our PCA (Fig. 1) and used a cold treatment starting at 2 h after dawn (ZT2). The up-regulation of genes with phases of ZT10 to ZT14 and the down-regulation of genes with phases of ZT18 to ZT2 are consistent with the negative and positive loadings for PC 1, respectively (Fig. 2). However, experiment j, not identified by unsupervised PCA, also showed a very similar pattern (Fig. 6), and this also used a cold treatment starting in the morning (4 h into a 16-h day). This clearly illustrates the benefit of experiment-wise supervised analysis. In general terms, the overrepresentation of repressed genes among those with phases immediately following dawn (ZT4–ZT8) is not specific, as it is observed in most experiments. In contrast, the genes with phases in the late night (ZT18–ZT22) are less consistent, being induced in some experiments and repressed in others. The lowest phase-specific regulation is seen for experiments D and e, where continuous light was used before and during cold treatment; however, there are differences between the two experiments and between the replicates within each experiment, and such replicate differences are less apparent in experiments performed in light-dark conditions (Supplemental Fig. S5). Nevertheless, the use of continuous light does not guarantee a low contribution of circadian genes, as experiment C, also using continuous light, shows strong phase bias and is very similar to the two experiments in which cold treatment was started in the middle of the day (Fig. 6).

DISCUSSION

Circadian and Diurnal Regulation Cause Variation in the Identity of Cold-Responsive Genes

Most studies to identify genes responding to cold state that measures to eliminate or minimize the effect of diurnal or circadian regulation were taken. Indeed, adequate precautions of starting and harvesting treatments at the same time of day are almost universally followed. Consequently, it is widely assumed that diurnal regulation is not a major source of variation between cold-responsive genes identified in different experiments. However, we demonstrate that diurnal- and circadian-regulated genes contribute most to the considerable differences between independent studies

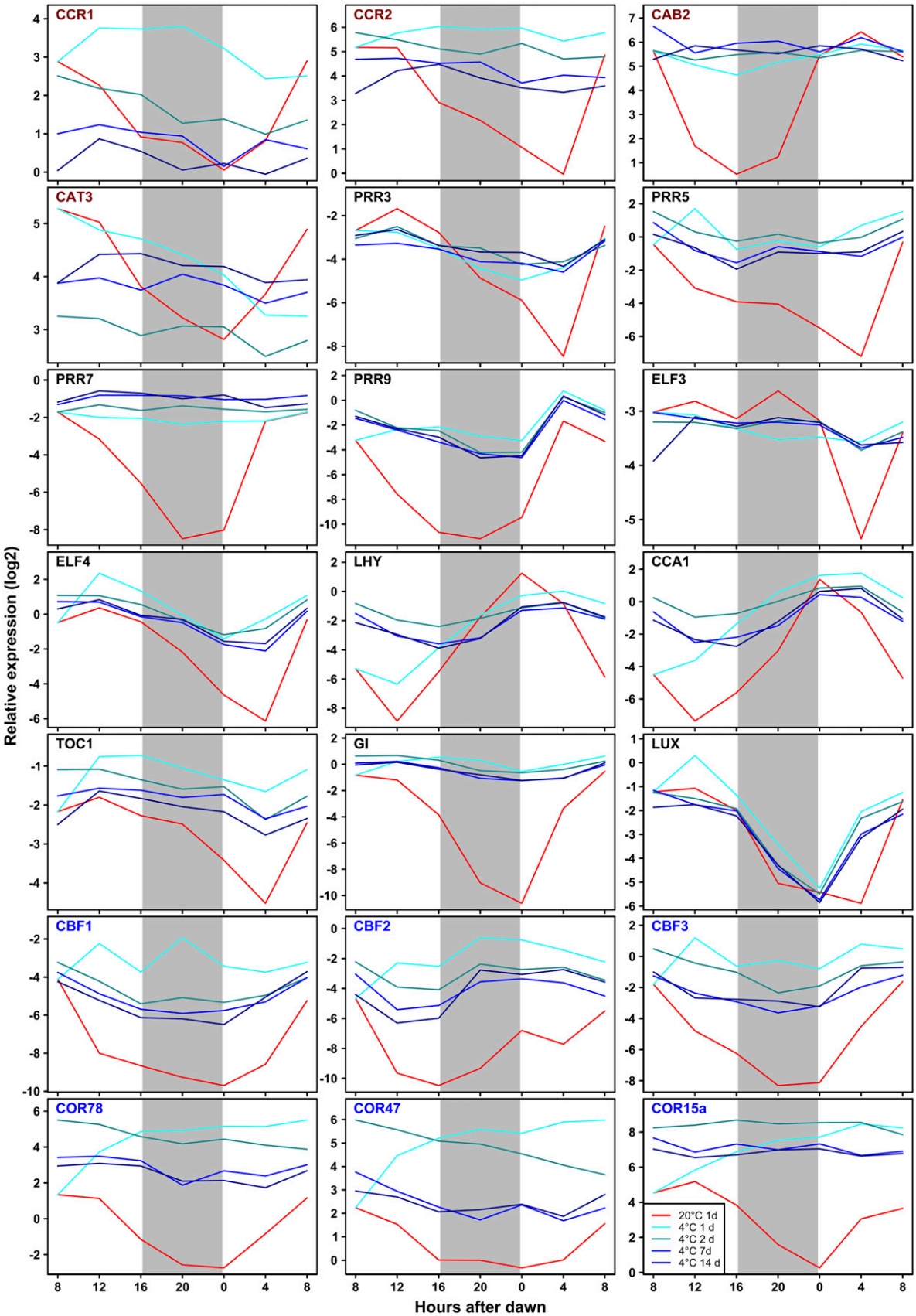

Figure 3. The oscillations of circadian clock components are dampened in light-dark cycles in the cold. Targeted expression

to identify genes responding to a 1-d cold treatment (Fig. 1; Table II). In addition, our targeted expression analyses explain why paired diurnal controls are insufficient to eliminate such variation. Following cold treatment, after a short initial response, most clock components and some output genes dampen to low-amplitude cycles, while other clock output genes stop to cycle (Fig. 3; Supplemental Fig. S3). Since genes in control samples show normal high-amplitude cycles, in samples taken at different times of the day diurnal- and circadian-regulated genes will make major contributions to those genes identified as cold responsive. Figure 7 schematically depicts these time-of-day effects on relative changes in gene expression between control and cold-treated plants. It can be easily appreciated why the time of day an experiment was started/harvested has such a large impact on the identity of cold-responsive genes. In this respect, experiments that were started in the morning (ZT2–ZT4) were separated from those starting at midday or in the evening.

Another large effect, similar to that of starting cold treatment in the morning, was found for the two experiments using diurnally grown plants and cold treatment performed in continuous light. Our targeted analysis again indicated an underlying reason for this: circadian oscillations are effectively stopped in the cold under continuous light (Fig. 4; Supplemental Fig. S4). The elimination of the oscillations that persist for some genes in light-dark cycles likely causes the apparent cold response to be further enhanced. This effect led to the previous suggestion that the higher expression of TOC1 (PRR1) and PRR5 after a 24-h cold treatment was the consequence of cold regulation rather than circadian effects (Lee et al., 2005; experiment B in our study), while our analyses reveal that this was a secondary effect of cold on the circadian clock in continuous light rather than a specific cold response. Furthermore, such secondary effects of cold on the circadian clock in continuous light also strongly influence the conclusions that may be drawn from the results of the AtGenExpress cold series (Kilian et al., 2007; experiment A in our study), which is currently the most widely used reference series for cold-responsive gene expression (Zimmermann et al., 2004; Toufighi et al., 2005). It is important to note that contrary to the assumptions of the authors (Kilian et al., 2007), circadian effects have a strong influence on the observed expression patterns.

Surprisingly, even the most extreme solution to eliminate diurnal regulation, using plants always grown in continuous light, does not guarantee the absence of circadian effects. Experiments D and e using continuous light did have the least circadian effects; however, there appeared to be an increased tendency for circadian phase differences between replicates (Supplemental Fig. S5). This could be caused by circadian oscillations, synchronized by either imbibition (Zhong et al., 1998) or stratification (Michael et al., 2003a), that were not actively considered or synchronized between experiments. In addition, strong phase-specific effects are seen for unknown reasons in experiment C, which also used continuous light. However, this could be a secondary effect, as sugar-regulated genes contributed strongly to the separation of this experiment by PC 2 (Table II; Supplemental Fig. S1). If the control plants in this experiment had low sugar levels, which would not be surprising under the unphysiological conditions of an agar plate, then increased sugar content due to cold treatment could make an enhanced contribution to gene regulation. Consistent with this explanation, experiment B (Fig. 1) was at the other extreme of the PC 2 separation, using agar supplemented with 3% Suc (Table I), resulting in a reduced contribution of sugar regulation.

Controlling and Understanding Cold-Diurnal Interactions

Microarray analysis has been used to dissect the contributions of factors, such as transcriptional regulators, cis-regulatory elements, functional annotations, and natural variation, to cold-responsive gene expression (Lee et al., 2005; Vogel et al., 2005; Hannah et al., 2006). In addition, the increasing availability of microarray data has fueled interest in meta-analysis (Hannah et al., 2005; Benedict et al., 2006) or online digital northern-blot expression analysis (Zimmermann et al., 2004; Toufighi et al., 2005) of the cold response. Our data show that time-of-day effects make significant contributions to the genes responding to cold identified in such studies; thus, a number of conclusions may be experiment dependent and should be regarded with some caution. Obviously, many conclusions will not be affected, as there are significant correlations of cold-responsive genes between experiments (Supplemental Table S1), but in the future a more explicit consideration of the effect of cold on diurnal and circadian oscillations will be necessary. Our data indicate that to control these effects, cold treatment should not involve a transfer from diurnal to continuous light; the timing of stratification/imbibition and harvest should be considered even in experiments using controls grown in continuous light; and that in all experiments, the possibility of

analysis for several circadian clock (black panel labels), circadian output (dark red panel labels), and cold-regulated (blue panel labels) genes was performed using qRT-PCR. Plants were grown under warm diurnal conditions under normal light in long days (16 h) before transfer to 4°C at 8 h after dawn. Whole rosettes were sampled from individual plants every 4 h across the 1st d in warm conditions and for days 1, 2, 7, and 14 in the cold. The *y* axes show raw expression (Ct; $\log_2$ scale) values normalized by subtracting the mean of three control genes. The *x* axes show time after dawn, with night shown in dark gray. Data are means from three biological replicate plants. SD values are not shown for clarity but averaged 0.3 Ct.

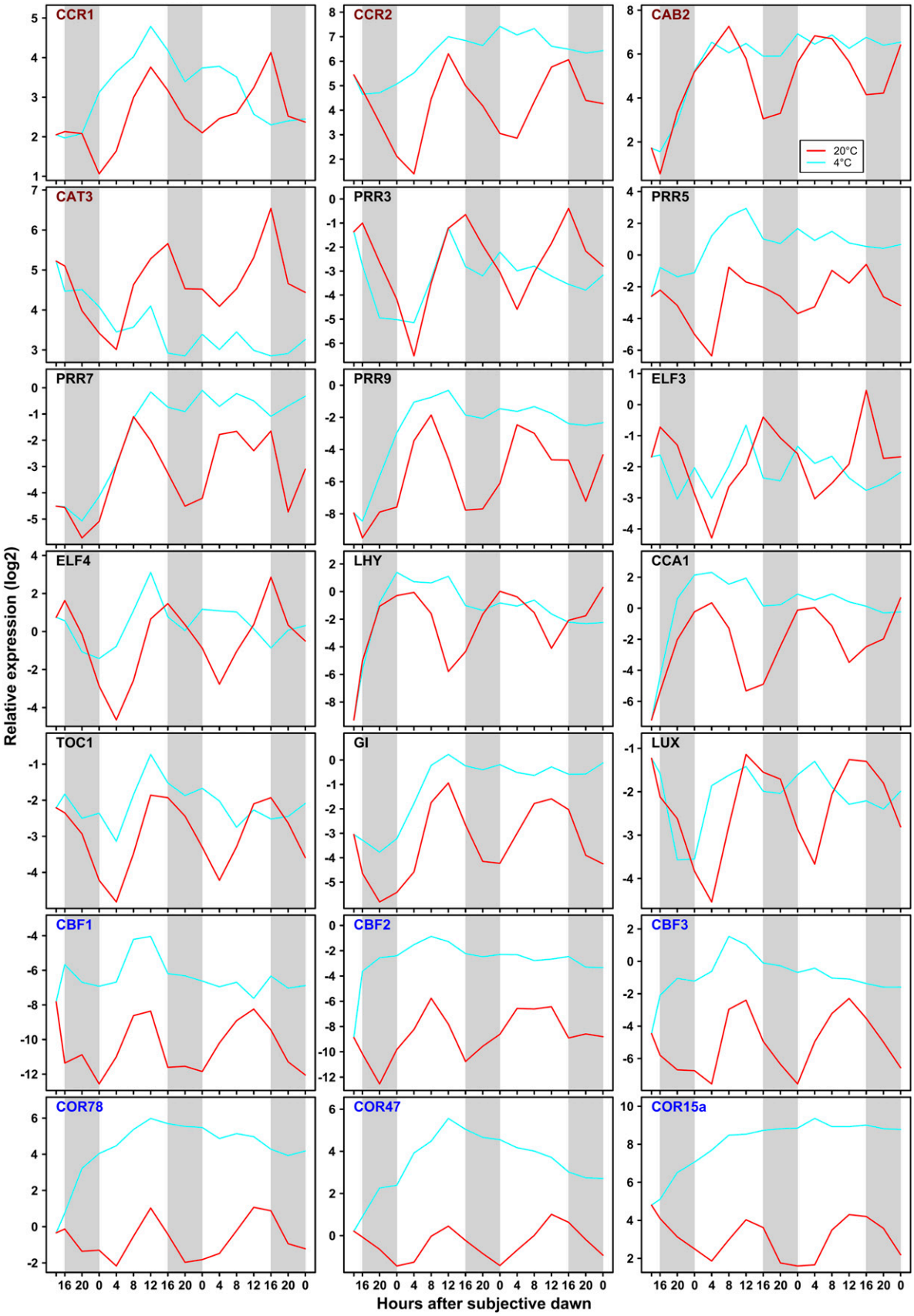

Figure 4. The oscillations of circadian clock components are stopped in continuous light in the cold. Targeted expression analysis

modified diurnal, sugar, and circadian regulation is considered prior to assigning "cold-specific" regulation.

Although cold-diurnal interactions have led to unintended differences in the identification of cold-responsive genes, it should also be considered that the short-term initial changes as well as the dampening or disruption of oscillations are all examples of cold regulation. Obviously, nonspecific effects of temperature on the thousands of chemical reactions within the cell will play a role in this effect. However, the normal amplitude oscillations of *LUX* and of many other genes (C. Espinoza, Z. Bieniawska, A. Leisse, L. Willmitzer, D.K. Hincha, and M.A. Hannah, unpublished data) indicate that plants can specifically avoid such general effects. Given the adaptive variation for circadian function (Michael et al., 2003b) and freezing tolerance (Hannah et al., 2006) and the fact that the circadian clock and low temperature both regulate thousands of genes, it could be beneficial for the plant to exploit a common mechanism for their regulation. In other words, if circadian oscillations are stopped by low temperature, it could be useful for the plant if a transcript is clamped to high, medium, or low expression levels, depending on its contribution to cold responses. Although the contribution of the circadian clock to plant fitness at warm temperatures has been demonstrated (Dodd et al., 2005), its necessity for plant growth, adaptation, or survival at low temperatures is completely unknown. Recent advances in the understanding of rhythmic diurnal versus circadian growth (Nozue et al., 2007), together with our data, suggest that understanding cold responses will require investigating diurnal as well as circadian regulation. Answers to this will await progress in two areas. First, molecular profiling of a regularly sampled diurnal time course in the cold is required to assess the extent and functional significance of the diurnal oscillations and expression changes that occur in the cold. Second, experiments assessing the growth, competitive advantage, and freezing tolerance of clock mutants and wild-type plants under different environmental conditions will be necessary to establish the functional significance of the underlying molecular mechanisms.

Diurnal Gating of the Cold Response

It was previously demonstrated that the circadian clock gates the cold induction of the *CBF1* to *CBF3*, *RAV1*, and *ZAT12* TFs (Fowler et al., 2005), $[Ca^{2+}]_{CYT}$ signals, and the expression of *COR78* (Dodd et al., 2006). Expression analysis of approximately 1,900 Arabidopsis TFs allowed us to generalize conclusions on their diurnal gating. First, and in common with our other analyses, it revealed a high dependence of the genes identified as cold regulated on the time of day the cold treatment started. Around 75% more TFs were cold induced in the morning than in the evening, and consistently, most of these cold-induced genes tended to reach higher absolute abundance after the morning cold treatment (Fig. 5). This was mostly due to higher cold induction rather than to higher initial abundance, which was often higher at ZT14 than at ZT2 (Fig. 5). Analysis of published data (Blasing et al., 2005) and our own data (Fig. 3; data not shown) indicated that many genes showed peak expression around midday in diurnal conditions. Therefore, induction increased similarly to the upturn in their normal cycles. The gating of a CBF2::GUS promoter-reporter fusion (Fowler et al., 2005) supports the involvement of transcriptional regulation in circadian gating of the cold response. However, given the number of gated TFs, it seems unlikely that they are all regulated by a single master TF. A more general mechanism could involve rhythmic changes in permissive chromatin structure that favor the induction of endogenously cycling genes, similar to the recently demonstrated regulation of *TOC1* transcription (Perales and Mas, 2007). An alternative or complementary mechanism to transcription-mediated changes in transcript abundance could involve the demonstrated circadian control of transcript stability (Lidder et al., 2005), possibly related to general cellular processes such as transcript degradation or ribosome occupancy. The generality of the low-temperature gating could imply that it is a nonspecific effect of the usual diurnal cycling of these transcripts, although this raises the circular argument of why these transcripts are diurnally regulated. Therefore, the physiological significance and downstream effects of diurnal gating will await a direct and thorough investigation of diurnal gating of downstream molecular changes and plant survival.

Cold Treatment as a Tool to Probe Clock Function

There has been much effort to understand the molecular basis of the circadian clock. This has culminated in the development of models of clock function that seek to explain existing data and direct new experiments (Locke et al., 2005, 2006; Zeilinger et al., 2006). Most of the current data describe clock function at warm temperatures often under continuous envi-

for several circadian clock (black panel labels), circadian output (dark red panel labels), and cold-regulated (blue panel labels) genes was performed using qRT-PCR. Plants were grown under warm diurnal conditions under low light in long days (16 h) before transfer to continuous light at 20°C or 4°C at 14 h after dawn. Whole rosettes were sampled from individual plants every 4 h until 58 h. The *y* axes show raw expression (Ct; $\log_2$ scale) values normalized by subtracting the mean of three control genes. The *x* axes show time after subjective dawn, with subjective night shown in light gray. Data are means from three biological replicate plants. SD values are not shown for clarity but averaged 0.5 Ct.

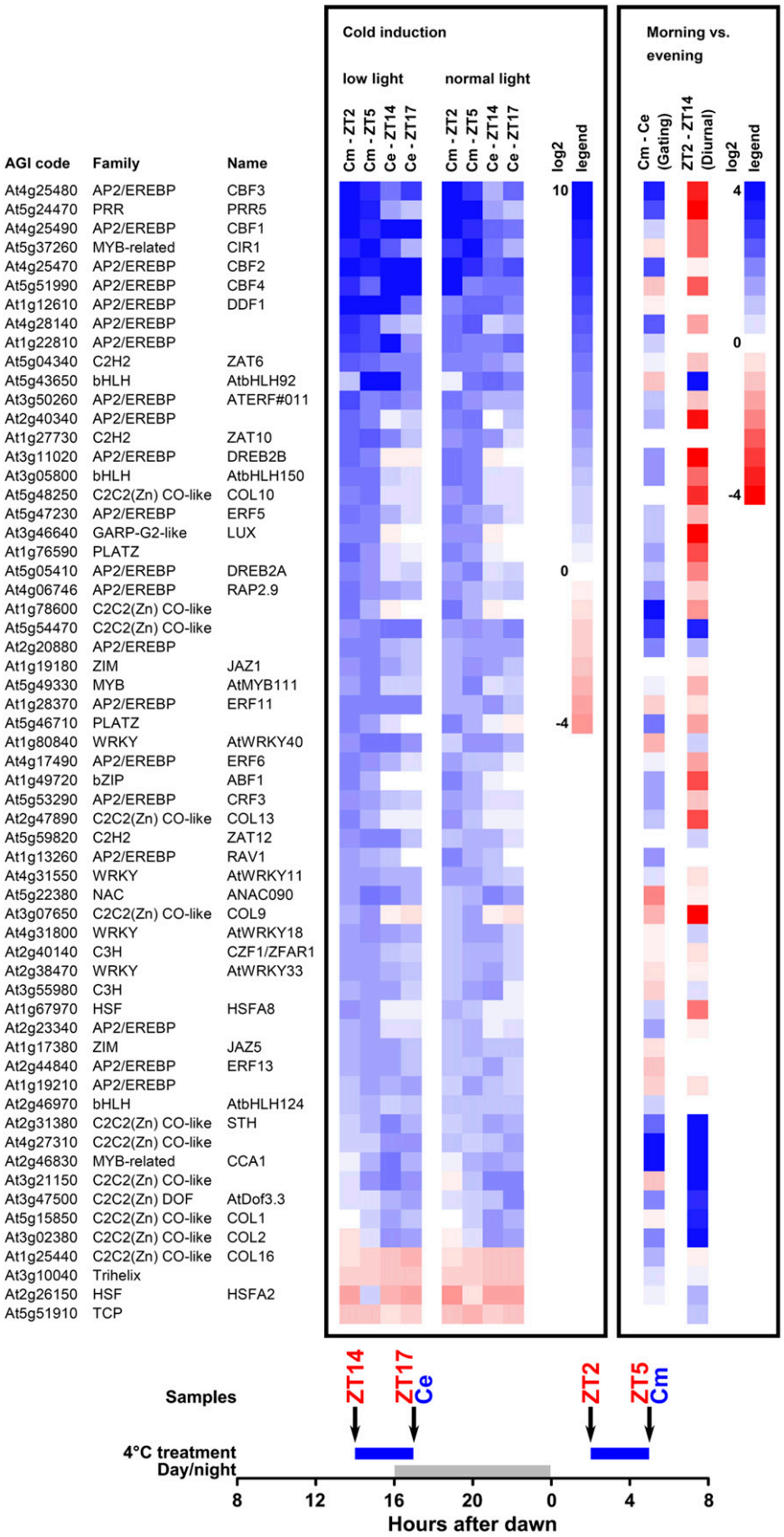

Figure 5. Diurnal gating of cold-responsive TFs. qRT-PCR for 1,880 Arabidopsis TFs was used to select strongly cold-responsive TFs (>4-fold change) using pooled samples from two independent experiments. Data are presented for the 60 TFs that were subsequently confirmed to change significantly using within-experiment biological replicates. Prior to the experiments, plants were grown under warm diurnal conditions at either low or normal light in long days (16 h). Plants were then transferred to 4°C (or simulated control transfer) at 2 h (ZT2) or 14 h (ZT14) after dawn. Whole rosettes were sampled from control plants before cold (ZT2 and ZT14), from paired diurnal controls (ZT5 and ZT17), or from plants cold treated for 3 h at ZT2 (Cm) or ZT14 (Ce). The sampling scheme and sample names are illustrated at the bottom. Only the 56 up-regulated and four down-regulated TFs that were significantly ($P < 0.05$, *t* test) induced at least 4-fold against both controls in both experiments for either ZT2 and/or ZT14 are shown. Normalized values were compared to generate $\log_2$ ratios between samples of interest, and these were used to plot heat maps. The left panel shows cold induction versus the time zero and paired control for each experiment, indicating gating of relative induction. The first column of the right panel shows absolute gating as the difference between the expression attained after morning cold treatment at ZT2 (Cm) versus evening cold treatment at ZT14 (Ce). The second column reveals diurnal regulation by the difference in expression between ZT2 and ZT14. Data are mean $\log_2$ ratios from five replicates.

Table III. *The induction of the CBF TFs is diurnally gated*

Expression of the CBF TFs was measured using qRT-PCR. Plants were grown under warm diurnal conditions under normal or low light in long days (16 h) before transfer to 4°C (or simulated control transfer) either at 2 h (ZT2) or 14 h (ZT14) after dawn. At both points, samples were harvested after 3 h at 4°C (Cm and Ce) and at 0 h (ZT2 and ZT14) and 3 h (ZT5 and ZT17) in control conditions. The sampling scheme is illustrated in Figure 5. Data are mean $\log_2$ ratios ($n = 5$). In low light, CBF1 was not detected at ZT2 generating infinite ratios (±inf).

CBF	Cold Induction								Morning Versus Evening			
	Low Light				Normal Light				Low Light		Normal Light	
	Cm–ZT2	Cm–ZT5	Ce–ZT14	Ce–ZT17	Cm–ZT2	Cm–ZT5	Ce–ZT14	Ce–ZT17	Cm–Ce	ZT2–ZT14	Cm–Ce	ZT2–ZT14
CBF1	+inf	8.3	10.5	9.7	8.5	8.4	5.7	5.2	−2.5	−inf	1.7	−1.1
CBF2	9.7	8.6	8.5	9.1	10.3	8.5	4.9	6.9	2.8	1.5	3.1	−2.3
CBF3	13.0	8.1	5.3	7.4	10.2	7.5	2.9	5.8	3.3	−4.4	4.0	−3.3

ronments, conditions in which clock components often show very similar expression patterns (Alabadi et al., 2001; Hazen et al., 2005). We hypothesized that as cold would likely affect the rates of transcription and transcript degradation pathways specifically as well as generally, then differential regulation of clock components would be revealed by their expression patterns in the cold. *TOC1* and *LUX* have similar expression in several conditions and are proposed to function closely together in the central oscillator (Hazen et al., 2005). Our experiments clearly distinguished the transcript regulation of these components. In light-dark cycles at 4°C, the cycles of *LUX* transcript were maintained at the same high amplitude, while similar to other clock components, *TOC1* showed low-amplitude oscillations clamped at high expression (Fig. 3; Supplemental Fig. S3). This indicates that either the rate of transcription or of transcript degradation of *TOC1* and *LUX* is distinct and highlights a potentially unique role for *LUX* among the identified clock components. A significant role for LUX was previously suggested as, unlike most oscillator components, the single loss-of-function mutant is arrhythmic in all measured outputs (Hazen et al., 2005; Onai and Ishiura, 2005).

Interestingly, cold rendered *LUX* expression immediately responsive following dawn, rather than with the 4-h delay observed under control conditions. *CCA1* and *LHY* peak around dawn and have been shown to bind an evening element in the *LUX* promoter; they may repress its transcription in a similar way to their regulation of *TOC1* (Hazen et al., 2005). Under our warm conditions, the diurnal amplitude of *CCA1* and *LHY* was between 250- and 2,000-fold, while in the cold peak abundance was similar for *CCA1* and 4- to 8-fold lower for *LHY*. The large effect on absolute *LHY* transcript quantity may explain the earlier induction of *LUX* via reduced LHY-mediated repression. However, relatively, the difference is small, and this explanation is not consistent with the tailing/delay in peak *LHY* and *CCA1* transcript or their overall increase in the cold, which should respectively further delay or repress *LUX*. In addition, the trough expression of the LHY/CCA1-repressed *TOC1* occurs at the same time in the warm or cold. Therefore, alternatively, these data might suggest that *LUX* is light regulated but that this regulation is usually gated by the circadian clock, probably via LHY/CCA1 repression. Obviously, other levels of regulation beyond transcription will have to be considered in the future, but the distinct regulation of *LUX* and *TOC1* revealed here will help direct experiments to test these hypotheses.

Progress in plant circadian research has been predominantly driven by the use of forward genetic screens to identify plants with aberrant expression of the circadian clock-regulated promoter-luciferase (LUC) fusion CAB2::LUC (Millar et al., 1995). A forward genetic screen for arrhythmic GI::LUC mutants has also been performed (Onai and Ishiura, 2005), and the circadian phenotypes of some CAB2::LUC mutants have been confirmed with a CCR2::LUC fusion (Hazen et al., 2005). The mutants that can be isolated using these reporters will depend on the regulation of the native promoter of the circadian output gene used. Measuring transcript abundance following cold treatment could reveal differences in the mechanisms underlying circadian regulation of clock output genes. Data from our targeted expression analysis support this. These showed that *CAB2*, *CCR2*, and *GI* show similar patterns of transcript abundance after cold treatment, clamping to near peak abundance (Fig. 4), while the expression of *CAT3* was significantly different, clamping to near minimum abundance. This may be consistent with a previous report describing the presence of two circadian oscillators in Arabidopsis, one preferentially light regulated and driving *CAB2* expression and the other preferentially responding to temperature and entraining *CAT3* expression (Michael et al., 2003a). An exciting prospect of a future genome-wide analysis of a cold diurnal time series would be the identification of sets of genes under differential mechanisms of circadian control. Furthermore, as the circadian clock appears to stop in the cold, the remaining oscillations likely reflect the daily input of light-dark cycles. Therefore, such data may also help light and circadian pathways to be separated and provide insight into circadian gating. Together, these data would likely assist in the identification of new reporter genes for forward genetic screens to provide further insight into mechanisms underlying the diurnal and circadian regulation of gene expression.

In conclusion, we show that although it is widely believed that diurnal and circadian effects on the iden-

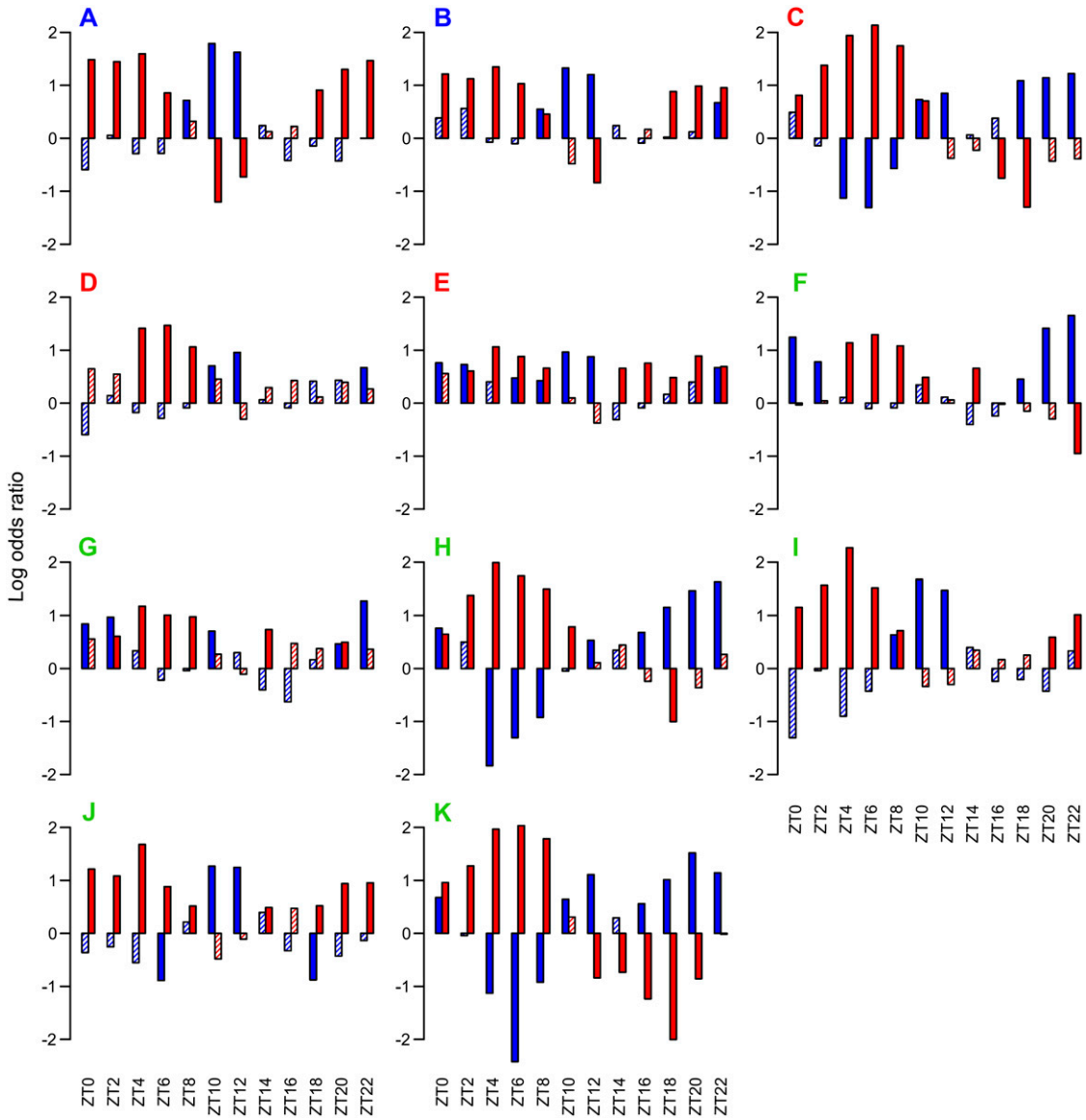

Figure 6. Experiment-specific bias in the cold response of circadian-regulated genes that peak at different phases of the day. The overlap between circadian-regulated genes that peak at different phases (Edwards et al., 2006) of the day (ZT, time after subjective dawn) and those responding to cold in independent studies (Table I) were compared. For direct comparability, we selected the 1,000 most induced (blue) and 1,000 most repressed (red) genes in each experiment and made the comparison using Fisher's exact test. Experiments are lettered as in Table I and labeled as in Figure 1; lowercase letters denote soil-grown plants. Colors indicate the light regime: red, continuous light for control and cold; blue, diurnal for control and continuous light for cold; green, diurnal for control and cold. The bars show the log odds ratios, which show whether the genes at a specific phase are more or less likely to be cold responsive than expected by chance. Significance (false discovery rate-corrected $P < 0.05$) is denoted by solidly colored bars, while nonsignificant log odd ratios are shown in hatched bars. [See online article for color version of this figure.]

tification of cold-responsive genes have been largely excluded through the use of paired controls, they account for the majority of differences between independent experiments to identify cold-responsive genes. Mechanistically, these differences in the cold are explained by the longer-term dampening of cycles for clock components and the stopping of the rhythmic expression of some output genes in light-dark cycles and arrhythmia of all cycles in continuous light. We also demonstrate that diurnal gating of cold-induced TFs is a general phenomenon and likely also contributes to observed differences. Diurnal regulation should thus be a key consideration of future experiments, and these should investigate its physiological significance for plant growth, adaptation, and survival in the cold. Finally, the differential effects of cold on *LUX* and on

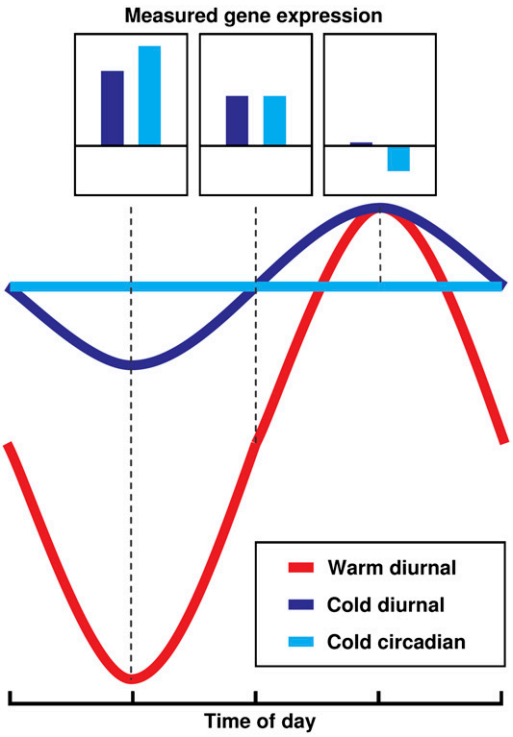

Figure 7. Simple model to illustrate the time-of-day effects on the identity of cold-responsive genes. In the cold, many genes, particularly of the core oscillator, show low-amplitude cycles in diurnal conditions, while in continuous light (circadian conditions) they stop to cycle. Therefore, even when paired controls are used, there are considerable time-of-day effects on measured gene expression changes. In reality, diurnal gating of gene expression, phase advances, and delays as well as the continued cycles of many genes mean that time-of-day influences will be much greater and more diverse than illustrated. [See online article for color version of this figure.]

circadian output genes suggest that low temperature could be an important tool to probe mechanisms underlying diurnal and circadian function.

MATERIALS AND METHODS

Plant Material and Growth

The protocols used were based on those we have described previously (Rohde et al., 2004; Hannah et al., 2005), with the exception that controlled-environment growth cabinets or chambers were used instead of a greenhouse. For all experiments, Arabidopsis (*Arabidopsis thaliana*) accession Columbia was initially grown on soil for 4 weeks in short days (8 h) before transfer to long days (16 h) at a day/night air temperature of 20°C/18°C and either 90 μmol m^{-2} s^{-1} (low light) or 150 μmol m^{-2} s^{-1} (normal light). Experiments were started when the rosette was mature (40–45 d after germination) and completed before the inflorescence reached 1 to 2 cm. Control plants were transferred to the same diurnal conditions or continuous light at the same intensity at 20°C. Cold treatment was always at an air temperature of 4°C and a light intensity of 90 μmol m^{-2} s^{-1}, but photoperiod was either 16 h or continuous. Treatments were started and samples harvested at the specified times for each experiment. Samples were harvested from individual plants and immediately frozen in liquid nitrogen before being powdered using either a ball mill (Retsch) or a cryogenic grinding robot (Labman Automation; Stitt et al., 2007).

Expression Analysis

qRT-PCR

Essentially, the protocols were similar to those described previously (Czechowski et al., 2004, 2005). Total RNA was extracted using Trizol reagent (Invitrogen) and treated with DNase (Roche or Ambion). RNA yield and quality were assessed using a nanodrop spectrophotometer (Nanodrop Technologies) and gel electrophoresis followed by qRT-PCR using an intron-specific primer (At5g65080) to confirm the absence of genomic DNA contamination. First-strand cDNA was synthesized from 2.5 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen), and its quality was assessed using primers that amplify 3′ and 5′ regions of GAPDH (At1g13440). Primers were mostly as published previously (Czechowski et al., 2004, 2005; Rohde et al., 2004; Morcuende et al., 2007) but are all summarized in Supplemental Table S3. qRT-PCR using SYBR Green to monitor double-stranded DNA synthesis was performed in an ABI PRISM 7900 HT 384-well plate Sequence Detection System (Applied Biosystems). Reactions contained 2.5 μL of 2× SYBR Green Master Mix reagent (Power SYBR Green [Applied Biosystems] or SYBR Green [Eurogentec]), 0.5 μL of cDNA (diluted 10- to 20-fold), and 2 μL of 0.5 μM primers. To ensure accuracy, primers were first added to each plate followed by a Mastermix containing the cDNA and SYBR Green, and both steps were performed using an Evolution P3 pipetting robot (Perkin-Elmer). RNA and cDNA quality control reactions were manually pipetted, and double volumes were used. In the diurnal and circadian time course experiments, Ct values for the genes of interest were normalized by subtracting the mean of three reference genes (At4g05320, At1g13320, and At2g32170; Czechowski et al., 2005). In the gating experiments, we used the same reference genes, but $\log_2$ ratios were generated after normalizing the expression for each TF using the scaling factor of the geNorm software (Vandesompele et al., 2002).

Expression Profiling

We performed expression profiling for experiments f, g, h, and j (Table I). All plants used had developed mature rosettes. In experiment f, 10-mm leaf discs were harvested from the tips of fully expanded leaves and samples were pooled from three plants, while in all other experiments, whole rosettes were harvested and pooled from five to six plants after grinding. Samples from experiments g and h were grown in parallel. Samples were hybridized to the Affymetrix ATH1 genome arrays (ATH1) at the German Resource Center for Genome Research or ATLAS Biolabs Berlin, as described previously (Hannah et al., 2005, 2006). However, for extraction, we used the RNeasy kit (Qiagen) following the manufacturer's instructions, and labeling was performed using the MessageAmpII kit (Ambion) using 1 μg of total RNA and 7 h of in vitro transcription. Expression data are available from the Arrayexpress database (accession nos. E-MEXP–1344 and E-MEXP–1345). All other expression data were obtained from public databases (Craigon et al., 2004; Barrett et al., 2007).

Raw CEL file data were analyzed using the bioconductor software project (Gentleman et al., 2004) to obtain GCRMA expression estimates (Wu et al., 2004) and MAS5 present/absent calls for each experiment. Values for the control samples were subtracted from the corresponding cold-treated sample values to give $\log_2$ differences. We retained 16,640 probe sets that were detected (Present/Absent call < 0.05) in any single experiment. For experiments with a single replicate, they had to be detected in both samples, while for replicated experiments, they had to be detected in either all control or all cold samples.

Data Analysis

Overrepresentation/underrepresentation analysis was performed using fisher.test and correlation analysis with cor in the R software. PCA was performed using the pcaMethods bioconductor package (Stacklies et al., 2007). The heat map (Fig. 5) was generated in Microsoft Excel using a macro kindly provided by Yves Gibon (Max-Planck-Institut für Molekulare Pflanzenphysiologie).

Microarray data from this article have been deposited with the European Bioinformatics Institute ArrayExpress data repository (http://www.ebi.ac.uk/arrayexpress/) under accession numbers E-MEXP-1344 and E-MEXP-1345.

Supplemental Data

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

Supplemental Figure S1. Suc-regulated genes make coordinate contributions to the separation of experiments by PC 2.

Supplemental Figure S2. Relationship between diurnal-, circadian-, and Suc-regulated genes overlapping with those contributing to variance between cold experiments.

Supplemental Figure S3. The oscillations of circadian clock components are dampened in light-dark cycles in the cold.

Supplemental Figure S4. The oscillations of circadian clock components are stopped in continuous light in the cold.

Supplemental Figure S5. Experiment and replicate-specific bias in the cold response of circadian-regulated genes that peak at different phases of the day.

Supplemental Table S1. The cold-responsive transcriptome shows significant correlation between independent experiments.

Supplemental Table S2. There are massive amounts of differences in cold-responsive genes between independent experiments.

Supplemental Table S3. Primers used in this study.

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LITERATURE CITED

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