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Transcriptional regulation of *LUX* by CBF1 mediates cold input to the circadian clock in Arabidopsis

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Summary

Circadian clocks allow organisms to anticipate daily changes in the environment to enhance overall fitness. Transcription factors (TFs) play a prominent role in the molecular mechanism but are incompletely described possibly due to functional redundancy, gene family proliferation, and/or lack of context-specific assays. To overcome these, we performed a high-throughput yeast one-hybrid screen using the *LUX ARRYHTHMO* (*LUX*) gene promoter as bait against an Arabidopsis TF library. *LUX* is a unique gene because its mutation causes severe clock defects and transcript maintains high amplitude cycling in the cold. We report the well-characterized cold-inducible C-repeat (CRT)/drought-responsive element (DRE) binding factor CBF1/DREB1b is a transcriptional regulator of *LUX*. We show that CBF1 binds the CRT in the *LUX* promoter, and both genes overlap in temporal and spatial expression. *CBF1* overexpression causes up-regulation of *LUX* and also alters other clock gene transcripts. *LUX* promoter regions including the CRT and Evening Element (EE) are sufficient for high amplitude transcriptional cycling in the cold, and cold-acclimated *lux* seedlings are sensitive to freezing stress. Our data show cold signaling is integrated into the clock by CBF-mediated regulation of *LUX* expression, thereby defining a new transcriptional mechanism for temperature input to the circadian clock.

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Results and Discussion

CBF1 binds the *LUX* promoter

LUX is a key component of the circadian clock regulating growth and development in Arabidopsis [1–6]. The *LUX* transcript is circadian-regulated with peak expression in the evening [1–2]. It also maintains high amplitude oscillations under diurnal conditions in the cold [7]. The clock proteins CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and REVEILLE8 (RVE8) regulate *LUX* promoter activity by binding the EE (Evening Element, AAAATATCT) motif [1,8], while the LBS (LUX binding site, GATA/TCG) mediates *LUX* auto-regulation [3]. TIMING OF CAB EXPRESSION1 (TOC1) also associates with the *LUX* promoter [9]. To identify other transcriptional regulators, we performed a yeast one-hybrid screen by challenging tiled *LUX* promoter fragments (Figure 1A) with an arrayed Arabidopsis transcription factor collection [10]. We found CBF1 (At4g25490) and CBF3 (At4g25480) strongly activated β-galactosidase (β-gal) reporter activity when the *LUX* promoter fragment −441/−222 was used as bait (Figure 1B). These factors are members of a small family of highly redundant AP2-domain transcription factors consisting of CBF1, CBF2 and CBF3 (also known as DREB1A-C) [11–15]. Although expressed under ambient conditions, cold treatment dramatically induces *CBF* expression and subsequent target genes to confer cold tolerance. The *LUX* promoter region −441/−222 possesses a single copy of the core C-repeat (CRT) element/dehydration responsive element (DRE) (CCGAC) at position −298/−294 [11,12,16,17]. This is specifically bound by CBF1 and CBF3 as mutation of 6bp including this core (−441/−222mutCRT) diminished β-gal activity (Figure 1B). An additional core CRT motif occurs at position −130/−126, however the CBFs were not recovered from our preliminary screen of the −253/−51 fragment nor in screens of the −660/−412 and −86/+156 regions that lack the CRT (data not shown). Although CBF2 was not recovered, the high functional redundancy between the CBFs suggests it might also bind the *LUX* promoter; additional studies are required to test this.

To confirm CBF binding *in vivo*, we generated transgenic seedlings overexpressing yellow fluorescent protein (YFP)-tagged CBF1 and performed chromatin immunoprecipitations (ChIPs) using a representative line (*CBF1ox2-1*). This line exhibits phenotypes similar to those previously published [12,15,18,19], which include an enhanced resistance to freezing stress (Figure S1). Chromatin was isolated from control and *CBF1ox2-1* seedlings grown under photocycles of 12-hours light/12-hours dark (abbreviated 12:12) at 22°C. In ChIP samples from control, specific binding was not detected at any tested regions including the negative control genes *UBIQUITIN* (*UBQ*) and *ACTIN* (*ACT*) (Figure 1C). In contrast, ChIP samples using *CBF1ox2-1* showed specific enrichment at the *COR78* promoter (amplicon −225/−144) that possesses three CRT motifs at positions −273/−268, −223/−218, −166/−161. Additionally enrichment was observed at the *LUX* promoter region −237/−129, which is flanked by CRTs at positions −298/−294 and −130/−126 (Figure 1A). Although the resolution of our ChIP experiments cannot distinguish between CRT sites, we confirmed CBF1 associates with the *LUX* promoter *in vivo*. Together with our yeast one-hybrid results, this indicates CBF1 occupies the *LUX* promoter by associating with the CRT motif.

CBF1 and *LUX* have overlapping expression patterns *in vivo*

To test whether CBF1 and CBF3 could regulate *LUX* expression *in vivo*, we compared expression patterns. Under ambient conditions, *CBF* expression is circadian with a peak phase in the afternoon [7, 20–22]. Using time-course array data from the Diurnal database [23], we compared expression under various diurnal and circadian conditions. *CBF1*, *CBF3* and *LUX* all cycled in 7 diurnal and 1 circadian datasets (Figure S2). All three genes overlapped in their temporal expression, and in many datasets the *CBFs* had phases preceding *LUX*. For subsequent analyses, we focused on *CBF1* as a representative of the *CBF* family. Using quantitative RT-PCR, we confirmed the sequential expression patterns of *CBF1* and *LUX* under constant light at 22°C (Figure 1D).

To determine whether spatial expression patterns overlap *in planta*, we generated GUS reporter lines driven by the *CBF1* and *LUX* promoters. The promoter fragments of *CBF1* and *LUX* were sufficient to drive GUS activity throughout tissues (Figure 1E–J). GUS activity was detected in cotyledons, rosette leaves, hypocotyls, roots, and root tips. Similar *CBF1* expression was reported previously [24]. The overlapping temporal and spatial expression patterns of *CBF1* and *LUX* are consistent with a regulatory interaction.

Overexpression of *CBF1* alters the levels of *LUX* and other clock gene transcripts

CBF regulation via the CRT motif causes activation of a suite of gene targets including the *CORs* [12,18]. To determine whether CBF1 regulates *LUX* expression, we analyzed the effect of *CBF1* overexpression in seedlings. First we tested whether *CBF1* overexpression alters the activity of a full-length *LUX* promoter-luciferase reporter (*LUX*::*LUC* −660/+156) but saw no obvious difference relative to control (data not shown). Since *CBF* overexpression reduces plant size [15, 25] and a control luciferase reporter for normalization is not available for Arabidopsis, it is possible a subtle effect is undetectable by this assay. We then measured *LUX* transcript levels in a previously published *CBF1* overexpression line [25]. This line expressed *CBF1*, *COR47*, and *COR78* at considerably higher levels than wildtype at all time-points tested (Figure 2). In contrast, the *LUX* transcript exhibited a significant increase only at specific times of the day near the wildtype peak (Figure 2). Additionally *PRR9*, *CCA1*, *LHY*, and *TOC1* showed significant alterations relative to the control. Unlike the *CORs*, the effects on *LUX* and the other clock genes are time specific. This may reflect their complex transcriptional regulation by multiple clock factors, and contribute to genetic robustness which is an inherent feature of circadian clocks that allows mutational perturbations to be accommodated without eliminating clock function [26]. As transcript patterns reflect the culmination of transcriptional and post-transcriptional events, other factors could also be involved. To confirm clock gene mis-regulation, we analyzed *LUX* transcripts in our *CBF1* overexpression line used in ChIP experiments. We observed a significant up-regulation of the *LUX* transcript in the subjective night at CT36 (circadian time 36) and time-specific alterations in other clock genes (Figure S3). The changes in clock gene dynamics are not identical between overexpression lines and may be due to differences in *CBF1* levels, ecotype backgrounds, and/or growth media. Nonetheless our observations support that *CBF1* overexpression impacts *LUX* and other clock gene transcripts.

The *LUX* promoter confers high amplitude oscillations in the cold

As the effects of *CBF1* overexpression on the *LUX* transcript are subtle and attempts to produce *CBF* triple knockout lines have been unsuccessful [27], we characterized the role of the CBFs in regulating the *LUX* promoter by using various LUC reporters. We first generated *LUX::LUC* lines that incorporated regions screened in our yeast one-hybrid assays (Figure 1A). Primary transgenic lines (T1) were imaged under constant light at 22°C. The full-length *LUX* promoter (−660/+156) was sufficient to drive circadian expression with a phase peak in the subjective evening similar to the endogenous *LUX* transcript (compare Figure 3A to Figure 2). Successive deletion of the 5' end caused small decreases in the overall expression as shown by comparing seedlings expressing fragment −660/+156 to those expressing −441/+156 and −253/+156. In contrast, further deletion caused a dramatic decrease in expression suggesting that the region spanning −253/−86 possesses regulatory elements important for transcriptional activation. Although other motifs may be involved, this decrease is consistent with the loss of EE and EE-like (EEL, AATATCT) motifs as the EE is sufficient for robust evening-phased expression [20,28].

We next selected representative single insertion T2 transgenic lines to compare relative expression under diurnal conditions at 22°C and 4°C. This cold condition was previously reported to dampen the amplitudes of all tested clock transcripts except *LUX* [7]. At 22°C, all *LUX* promoter fragments produced high amplitude oscillations. Progressive truncation of the *LUX* promoter had little effect on amplitude at 22°C (Figure 3B). As seen in Figure 3C, the full-length *LUX::LUC* construct recapitulated the reported transcript oscillations in the cold indicating the *LUX* promoter is sufficient to drive transcript cycling under these conditions. Interestingly we also noticed a small phase delay in expression after transfer to 4°C. This was reported previously for the transcript showing that the activity of the *LUX* promoter closely matches that of the endogenous gene [7]. After transfer to 4°C, deletion of the −600/−86 region caused a decrease in amplitude relative to the other fragments. Since this deletion removed both CRTs within the *LUX* promoter, this supports they may be required for the regulation of *LUX* expression in the cold.

To determine the minimal motifs sufficient for recapitulating the expression pattern of the full length *LUX* promoter in the cold, we generated reporters driven by short wildtype and mutant *LUX* sequences. The CRT at position −298/−294 is hypothesized to be important since CBF1 associates with the *LUX* promoter in yeast one-hybrid and ChIP assays (Figures 1B and 1C). As the *LUX* transcript oscillates and is evening-phased even though *CBF* transcripts are clamped high in the cold [7], the EE also appears important. CCA1 and RVE8 regulate *LUX* via the EE [1,8], and the EE and EEL are required for cold induction of *COL1* and *COR27* [29]. The *LUX* promoter possesses one EE and two EELs (Figure 1A). We cloned wildtype and mutant versions of the CRT (from position −298/−294) and EE in tandem to generate CRT+EE, mutant CRT+EE (mutCRT+EE), and CRT+mutant EE (CRT +mutEE) fragments. Primary transgenic lines were imaged at 22°C or 4°C. Because very few lines carrying the CRT+mutEE construct had detectable LUC levels at either temperature, they were omitted from analysis (data not shown). This lack of expression could be due to disrupted binding of circadian activators such as RVE8 [28] and cold regulators [29]. Transgenic plants carrying the CRT+EE construct displayed rhythmic

oscillations at 22°C (Figure 3D). Peak expression occurred at the end of the light period similar to that reported previously for other EE lines [20,28]. Mutation of the CRT had no effect on this pattern at 22°C, confirming the EE is sufficient for rhythmic oscillations under ambient conditions. When seedlings carrying the CRT+EE were transferred to 4°C, high amplitude rhythms were observed, with the first peak after transfer showing higher amplitude than at 22°C (Figure 3E). This suggests the CRT+EE responds to the cold but this response is gated to the evening. The phase of expression in the cold was also slightly delayed relative to that at 22°C. This is reminiscent of the pattern observed with the full length *LUX* promoter (Figure 3C) as well as the endogenous transcript expression pattern reported previously [7]. High amplitude cycling at 4°C is specifically dependent on the CRT since its mutation greatly reduced evening-phased expression (Figure 3E). These results indicate that the CRT, in combination with the EE, recruits activators necessary for evening-phased expression at 4°C.

While the EE and CRT are sufficient to recapitulate the expression of the endogenous *LUX* promoter in the cold, the full complement of factors regulating *LUX* expression remains unknown. Based on our and other results, we speculate the CBFs (via the CRT) and CCA1/LHY/RVE8 (via the EE) are pivotal at cooler temperatures. Simplistically under ambient conditions, the clock proteins CCA1/LHY/RVE8 confer time-of-day information with CCA1/LHY repressing *LUX* expression during the early-morning and RVE8 activating evening-phased expression. Since the CBFs are also expressed under ambient conditions, they likely contribute to *LUX* expression but play a lesser role as suggested by the subtle effects of *CBF1* overexpression (Figure 2). As temperatures decrease however, the expression of *CCA1/LHY* (and other clock genes) loses rhythmicity while *LUX* maintains high amplitude oscillations. Parallel to this, the expression of the *CBFs* increases with cold. We hypothesize as temperatures decrease and the oscillations of clock genes like *CCA/LHY* dampen, the CBFs play an increasingly important role to ensure *LUX* oscillations are maintained over a broad temperature range. However as *CBF* rhythms also dampen in the cold, post-transcriptional regulation and/or additional factors are also likely involved.

Freezing tolerance is disrupted in *lux* mutants

The cold response is a well-characterized output of the clock. CCA1 and LHY regulate freezing tolerance by directly associating with *CBF* promoter regions to gate their expression [30,31]. Loss of *CCA1/LHY* impairs freezing tolerance [30–32] while *CCA1* overexpression promotes freezing tolerance [33]. In addition, mutation of *PRR9/7/5* and *TOC1* enhances freezing tolerance, and PRR5, PRR7, and TOC1 associate with *CBF* promoters [9,34–37]. While the mechanism is unknown, loss of *GIGANTEA* (*GI*) also causes freezing sensitivity but independently of changes in *CBF* expression [38].

To test the functional relevance of *LUX* in the cold, we assessed the response of *lux* seedlings to freezing stress after cold acclimation (Figure 4). Plants were cold acclimatized before exposure to −5°C for 5 hours. Strikingly most *lux-1 and lux-4* seedlings died while the majority of control seedlings survived (Figure 4). This demonstrates that *LUX* contributes to freezing tolerance. Recently the *lux-2* mutant was reported to have similar sensitivity as wildtype to freezing stress in the absence of cold-acclimation [37]. Since the

lux-1, *lux-2*, and *lux-4* mutations all cause premature stop codons and affect clock processes similarly [1], this suggests *LUX* may have a very specific role during the acclimation process.

How LUX contributes to freezing tolerance remains to be determined. Under ambient conditions, *lux* has low and high expression of *CCA/LHY* and TOC1, respectively [1,2]. Since CCA1/LHY activates and TOC1 represses *CBF* expression, it is possible that the freezing sensitivity of *lux* is indirectly due to their regulation of *CBF* and target gene expression [31,37]. The *lux* mutant also exhibits high *GI* expression [2] but as the mechanism of *GI* involvement is unknown, this observation is not simple to resolve. Because LUX is a DNA-binding transcription factor, we speculate it may also directly regulate genes involved in cold tolerance. LUX functions in a transcriptional complex with other evening-phased proteins including ELF3 [4,6]. ELF3 was recently reported to associate with the *CBF3* promoter [37] so it is possible LUX also directly regulates *CBF3*. It was previously reported that the *lux* mutant under ambient conditions exhibits high *CBF1/3* expression [37]. However, we observed *lux-1* and *lux-4* are sensitive to freezing after cold-acclimation (Figure 4). Together this suggests *LUX* may regulate cold response genes in parallel to the *CBF* pathway. Determining the direct target genes of LUX may help clarify the mechanisms involved.

Like light sensing, we anticipate cold input to the Arabidopsis clock occurs through multiple molecular mechanisms. We have shown that the well-characterized cold-inducible CBF1 transcription factor plays an important role in maintaining *LUX* oscillations in the cold and that *LUX* plays an essential role in freezing tolerance after cold-acclimation. In addition to *LUX*, the CBFs could regulate other clock genes; for example the *LHY* and *TOC1* promoters contain CRT motifs and their transcripts are altered in *CBF1* overexpressing lines (Figures 2 and S3). Another mode of cold sensing in Arabidopsis occurs post-transcriptionally via alternative splicing of *CCA1* [33]. As the alternative *CCA1* transcript presumably encodes a protein that interferes with the full-length protein, its suppression is necessary for freezing tolerance. In other organisms, post-transcriptional mechanisms are common for cold input to the clock. Thermosensitive splicing of the Drosophila clock gene *period* affects clock phasing and circadian-regulated locomotor activity [39,40]. The Neurospora clock gene *FREQUENCY* (*FRQ*) also undergoes alternative splicing in a temperature-dependent manner to affect free-running rhythms and temperature compensation [41]. In addition, *FRQ* translation biases towards noncoding upstream open reading frames in the 5'UTR in order to restrict translation under decreasing temperatures [42,43]. Non-optimal codon usage for *FRQ* was also reported, and in cyanobacteria this mechanism limits translation of the central clock KaiB and KaiC proteins under cooler temperatures [44,45]. Finally in mouse, the cold-inducible RNA-binding protein (CIRP) is required for circadian rhythms in fibroblasts and directly regulates mRNA stability of the circadian oscillator *CLOCK* gene [46]. Our discovery that *LUX* is transcriptionally regulated by CBF1 adds a new layer of regulation to cold input to the circadian clock. Uncovering additional mechanisms of connectivity between temperature and the circadian clock will undoubtedly further our understanding of growth and development.

Experimental Procedures

For detailed protocols, see Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hazen SP, Schultz TF, Pruneda-Paz JL, Borevitz JO, Ecker JR, Kay SA. LUX ARRHYTHMO encodes a Myb domain protein essential for circadian rhythms. Proc Natl Acad Sci U S A. 2005; 102:10387–10392. [PubMed: 16006522]
2. Onai K, Ishiura M. PHYTOCLOCK 1 encoding a novel GARP protein essential for the Arabidopsis circadian clock. Genes Cells. 2005; 10:963–972. [PubMed: 16164597]
3. Helfer A, Nusinow DA, Chow BY, Gehrke AR, Bulyk ML, Kay SA. LUX ARRHYTHMO encodes a nighttime repressor of circadian gene expression in the Arabidopsis core clock. Curr Biol. 2011; 21:126–133. [PubMed: 21236673]
4. Nusinow DA, Helfer A, Hamilton EE, King JJ, Imaizumi T, Schultz TF, Farre EM, Kay SA. The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature. 2011; 475:398–402. [PubMed: 21753751]
5. Chow BY, Helfer A, Nusinow DA, Kay SA. ELF3 recruitment to the PRR9 promoter requires other Evening Complex members in the Arabidopsis circadian clock. Plant Signal Behav. 2012; 7:170–173. [PubMed: 22307044]
6. Herrero E, Kolmos E, Bujdoso N, Yuan Y, Wang M, Berns MC, Uhlworm H, Coupland G, Saini R, Jaskolski M, Webb A, Goncalves J, Davis SJ. EARLY FLOWERING4 recruitment of EARLY FLOWERING3 in the nucleus sustains the Arabidopsis circadian clock. Plant Cell. 2012; 24:428–443. [PubMed: 22327739]
7. Bieniawska Z, Espinoza C, Schlereth A, Sulpice R, Hincha DK, Hannah MA. Disruption of the Arabidopsis circadian clock is responsible for extensive variation in the cold-responsive transcriptome. Plant Physiol. 2008; 147:263–279. [PubMed: 18375597]
8. Hsu PY, Devisetty UK, Harmer SL. Accurate timekeeping is controlled by a cycling activator in Arabidopsis. Elife. 2013; 2:e00473. [PubMed: 23638299]
9. Huang W, Perez-Garcia P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P. Mapping the core of the Arabidopsis circadian clock defines the network structure of the oscillator. Science. 2012; 336:75–79. [PubMed: 22403178]
10. Pruneda-Paz JL, Breton G, Para A, Kay SA. A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. Science. 2009; 323:1481–1485. [PubMed: 19286557]
11. Stockinger EJ, Gilmour SJ, Thomashow MF. Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci U S A. 1997; 94:1035–1040. [PubMed: 9023378]
12. Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell. 1998; 10:1391–1406. [PubMed: 9707537]

13. Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF. Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J. 1998; 16:433–442. [PubMed: 9881163]
14. Medina J, Bargues M, Terol J, Perez-Alonso M, Salinas J. The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression Is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiol. 1999; 119:463–470. [PubMed: 9952441]
15. Gilmour SJ, Fowler SG, Thomashow MF. Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol Biol. 2004; 54:767–781. [PubMed: 15356394]
16. Baker SS, Wilhelm KS, Thomashow MF. The 5'-region of Arabidopsis thaliana cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol. 1994; 24:701–713. [PubMed: 8193295]
17. Yamaguchi-Shinozaki K, Shinozaki K. A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell. 1994; 6:251–264. [PubMed: 8148648]
18. Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science. 1998; 280:104–106. [PubMed: 9525853]
19. Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 2000; 124:1854–1865. [PubMed: 11115899]
20. Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA. Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science. 2000; 290:2110–2113. [PubMed: 11118138]
21. Edwards KD, Anderson PE, Hall A, Salathia NS, Locke JC, Lynn JR, Straume M, Smith JQ, Millar AJ. FLOWERING LOCUS C mediates natural variation in the high-temperature response of the Arabidopsis circadian clock. Plant Cell. 2006; 18:639–650. [PubMed: 16473970]
22. Kidokoro S, Maruyama K, Nakashima K, Imura Y, Narusaka Y, Shinwari ZK, Osakabe Y, Fujita Y, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K. The phytochrome-interacting factor PIF7 negatively regulates DREB1 expression under circadian control in Arabidopsis. Plant Physiol. 2009; 151:2046–2057. [PubMed: 19837816]
23. Mockler TC, Michael TP, Priest HD, Shen R, Sullivan CM, Givan SA, McEntee C, Kay SA, Chory J. The DIURNAL project: DIURNAL and circadian expression profiling, model-based pattern matching, and promoter analysis. Cold Spring Harb Symp Quant Biol. 2007; 72:353–363. [PubMed: 18419293]
24. Novillo F, Medina J, Salinas J. Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. Proc Natl Acad Sci U S A. 2007; 104:21002–21007. [PubMed: 18093929]
25. Achard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P. The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell. 2008; 20:2117–2129. [PubMed: 18757556]
26. Hogenesch JB, Ueda HR. Understanding systems-level properties: timely stories from the study of clocks. Nat Rev Genet. 2011; 12:407–416. [PubMed: 21556016]
27. Gery C, Zuther E, Schulz E, Legoupi J, Chauveau A, McKhann H, Hincha DK, Teoule E. Natural variation in the freezing tolerance of Arabidopsis thaliana: effects of RNAi-induced CBF depletion and QTL localisation vary among accessions. Plant Sci. 2011; 180:12–23. [PubMed: 21421342]
28. Harmer SL, Kay SA. Positive and negative factors confer phase-specific circadian regulation of transcription in Arabidopsis. Plant Cell. 2005; 17:1926–1940. [PubMed: 15923346]
29. Mikkelsen MD, Thomashow MF. A role for circadian evening elements in cold-regulated gene expression in Arabidopsis. Plant J. 2009; 60:328–339. [PubMed: 19566593]
30. Fowler SG, Cook D, Thomashow MF. Low temperature induction of Arabidopsis CBF1, 2, and 3 is gated by the circadian clock. Plant Physiol. 2005; 137:961–968. [PubMed: 15728337]

31. Dong MA, Farre EM, Thomashow MF. Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in Arabidopsis. Proc Natl Acad Sci U S A. 2011; 108:7241–7246. [PubMed: 21471455]
32. Espinoza C, Degenkolbe T, Caldana C, Zuther E, Leisse A, Willmitzer L, Hincha DK, Hannah MA. Interaction with diurnal and circadian regulation results in dynamic metabolic and transcriptional changes during cold acclimation in Arabidopsis. PLoS One. 2010; 5:e14101. [PubMed: 21124901]
33. Seo PJ, Park MJ, Lim MH, Kim SG, Lee M, Baldwin IT, Park CM. A self-regulatory circuit of CIRCADIAN CLOCK-ASSOCIATED1 underlies the circadian clock regulation of temperature responses in Arabidopsis. Plant Cell. 2012; 24:2427–2442. [PubMed: 22715042]
34. Nakamichi N, Kusano M, Fukushima A, Kita M, Ito S, Yamashino T, Saito K, Sakakibara H, Mizuno T. Transcript profiling of an Arabidopsis PSEUDO RESPONSE REGULATOR arrhythmic triple mutant reveals a role for the circadian clock in cold stress response. Plant Cell Physiol. 2009; 50:447–462. [PubMed: 19131357]
35. Nakamichi N, Kiba T, Kamioka M, Suzuki T, Yamashino T, Higashiyama T, Sakakibara H, Mizuno T. Transcriptional repressor PRR5 directly regulates clock-output pathways. Proc Natl Acad Sci U S A. 2012; 109:17123–17128. [PubMed: 23027938]
36. Liu T, Carlsson J, Takeuchi T, Newton L, Farre EM. Direct regulation of abiotic responses by the Arabidopsis circadian clock component PRR7. Plant J. 2013; 76:101–114. [PubMed: 23808423]
37. Keily J, Macgregor DR, Smith RW, Millar AJ, Halliday KJ, Penfield S. Model selection reveals control of cold signalling by evening-phased components of the plant circadian clock. Plant J. 2013; 76:247–257. [PubMed: 23909712]
38. Cao S, Ye M, Jiang S. Involvement of GIGANTEA gene in the regulation of the cold stress response in Arabidopsis. Plant Cell Rep. 2005; 24:683–690. [PubMed: 16231185]
39. Cheng Y, Gvakharia B, Hardin PE. Two alternatively spliced transcripts from the Drosophila period gene rescue rhythms having different molecular and behavioral characteristics. Mol Cell Biol. 1998; 18:6505–6514. [PubMed: 9774666]
40. Majercak J, Sidote D, Hardin PE, Edery I. How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron. 1999; 24:219–230. [PubMed: 10677039]
41. Garceau NY, Liu Y, Loros JJ, Dunlap JC. Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell. 1997; 89:469–476. [PubMed: 9150146]
42. Liu Y, Garceau NY, Loros JJ, Dunlap JC. Thermally regulated translational control of FRQ mediates aspects of temperature responses in the Neurospora circadian clock. Cell. 1997; 89:477–486. [PubMed: 9150147]
43. Diernfellner AC, Schafmeier T, Merrow MW, Brunner M. Molecular mechanism of temperature sensing by the circadian clock of Neurospora crassa. Genes Dev. 2005; 19:1968–1973. [PubMed: 16107616]
44. Zhou M, Guo J, Cha J, Chae M, Chen S, Barral JM, Sachs MS, Liu Y. Non-optimal codon usage affects expression, structure and function of clock protein FRQ. Nature. 2013; 495:111–115. [PubMed: 23417067]
45. Xu Y, Ma P, Shah P, Rokas A, Liu Y, Johnson CH. Non-optimal codon usage is a mechanism to achieve circadian clock conditionality. Nature. 2013; 495:116–120. [PubMed: 23417065]
46. Morf J, Rey G, Schneider K, Stratmann M, Fujita J, Naef F, Schibler U. Cold-inducible RNA-binding protein modulates circadian gene expression posttranscriptionally. Science. 2012; 338:379–383. [PubMed: 22923437]

Highlights

- The *LUX* clock gene promoter is bound by the cold-induced CBF1 transcription factor
- *CBF1* overexpression up-regulates *LUX* and alters other clock gene transcripts
- The CRT and EE motifs are sufficient to confer evening-phased expression in the cold
- *lux* mutants are sensitive to freezing after cold-acclimation

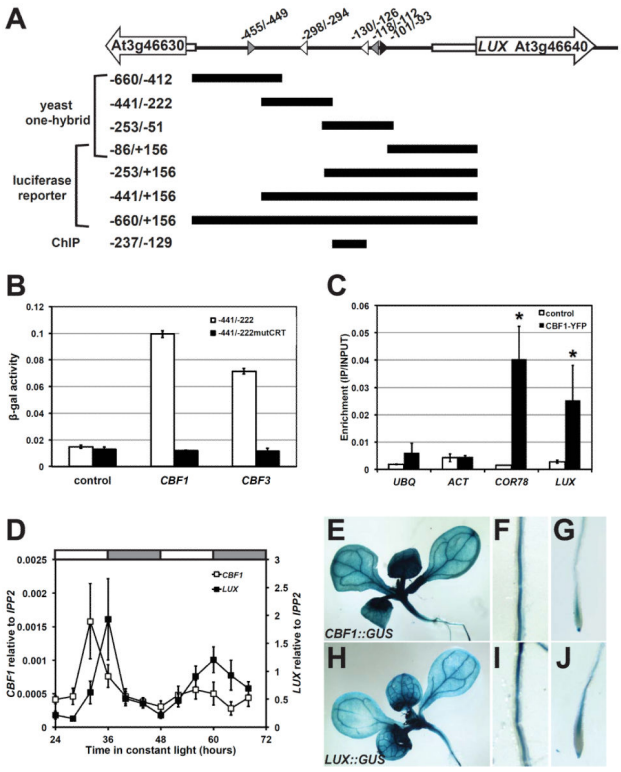

Figure 1.

CBF1 binds the *LUX* promoter

(A) Schematic of the *LUX* promoter. The transcriptional start occurs at +1. White arrows: gene bodies; white rectangles: 5'UTRs; arrowheads: DNA motifs with EELs in grey, CRTs in white, and EE in black; and black bars: promoter regions tested in different assays. Positions and sizes are roughly drawn to scale. (B) CBF1 binding to *LUX* promoter in yeast. Bars represent β-galactosidase activity where error bars represent standard error of three replicates. (C) CBF1 binding to the *LUX* promoter in Arabidopsis. ChIP assays were performed using control (*LUX::LUC* −660/+156 line 139) or *CBF1* overexpressing line 2–1 (*CBF1ox2-1*). Plants were grown under 12:12 and collected 16 hours after lights on for ChIP using an anti-GFP antibody. Results are normalized to input DNA. Bars represent average quantification from real-time PCR with error bars representing standard error of two independent experiments. Student's t-test was used to determine the significance of target enrichment relative to control (* p-value ≤0.05). *UBQ*, *UBIQUITIN*; and *ACT*, *ACTIN*. (D) Temporal expression of *CBF1* and *LUX*. Wildtype plants were entrained under 12:12 before release to constant light at 22°C. mRNA levels were quantified by real-time reverse transcription PCR and normalized to *IPP2*. Data is the average of three biological replicates with error bars representing standard error. (E–J) Spatial expression of *CBF1* and *LUX*. Seedlings carrying promoter::GUS constructs of *CBF1* (E–G) and *LUX* (H–J) were grown under 12:12 at 22°C. Expression detected throughout cotyledons, rosette leaves and hypocotyl (E and H); roots (F and I); and root tips (G and J).

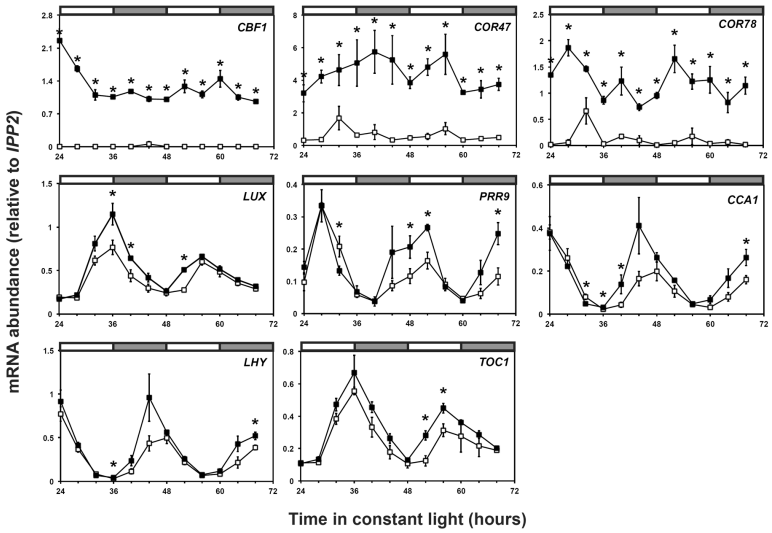

Figure 2.

CBF1 overexpression affects *LUX* and other clock gene transcripts

Transcript abundance was measured in control (open squares) and *CBF1* overexpressor line (filled squares). Seedlings were entrained under 12:12 at 22°C for 10 days before transfer to constant light. Transcript levels were quantified by real-time reverse transcription PCR. Data is the average of three biological replicates with error bars representing standard error. Student's t-test was used to determine the significance of transcript levels relative to control (* p-value ≤0.05).

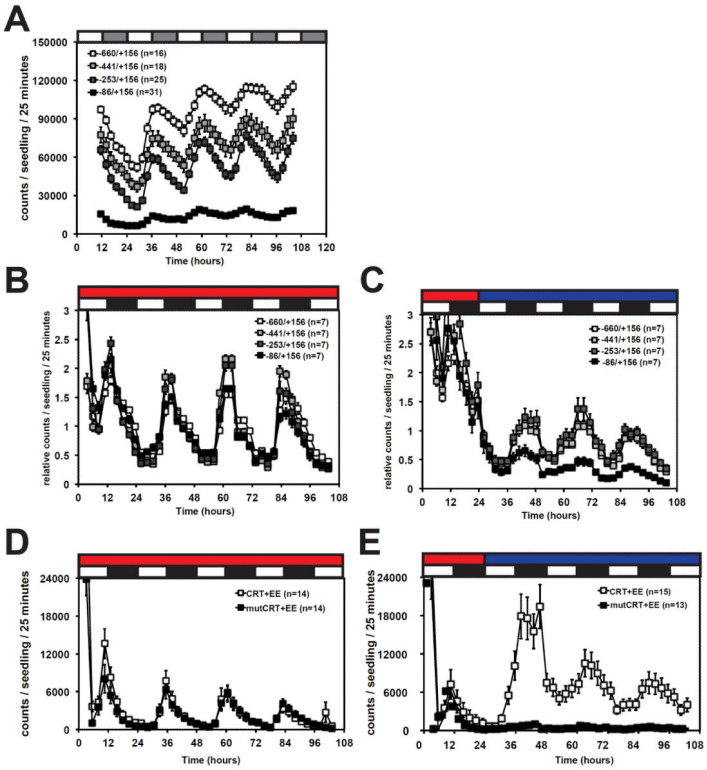

Figure 3.

The CRT and EE in the *LUX* promoter confer evening-phased oscillations in the cold Bioluminescence assays in Arabidopsis seedlings carrying luciferase reporters driven by *LUX* promoter fragments (A–C) or multimerized CRT and EE motifs (D and E). Ten-day old seedlings were entrained under 12:12 at 22°C. Seedlings remained under these conditions (B and D) or were transferred to constant light at 22°C (A) or 12:12 at 4°C (C and E). Red and blue bars represent 22°C and 4°C respectively. Seedlings in the T1 (A,D,E) or T2 (B,C) generation were assayed. Data shown is the averages of all plants imaged in a given experiment with error bars representing standard error. Values in B and C are relative to the average expression of that reporter line. Traces are representative of data from at least two independent experiments.

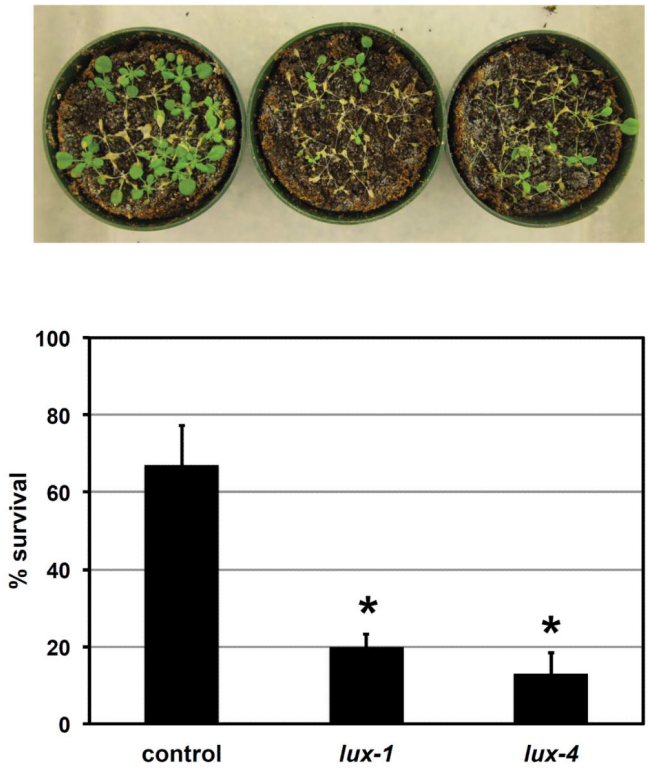

Figure 4.

LUX is required for survival to freezing stress after cold acclimation

Freezing tolerance is expressed as percent (%) survival. Control (*CAB2*::*LUC*), *lux-1*, and *lux-4* seedlings were grown under 12:12 at 22°C for approximately 4 weeks. Plants were then transferred at 4 hours after lights on into 4°C darkness for 3 days cold acclimation before freezing at −5°C for 5 hours. After 24 hours recovery at 4°C, plants were returned to 12:12 at 22°C for 7 days. Data is the average of four independent experiments with error bars representing standard error. Student's t-test was used to determine the significance of survival relative to control (* p-value ≤0.05).