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Report

LUX ARRHYTHMO Encodes a Nighttime Repressor of Circadian Gene Expression in the *Arabidopsis* Core Clock

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Summary

Circadian clocks provide an adaptive advantage by allowing organisms to anticipate daily and seasonal environmental changes [1, 2]. Eukaryotic oscillators rely on complex hierarchical networks composed of transcriptional and posttranslational regulatory circuits [3]. In *Arabidopsis*, current representations of the circadian clock consist of three or four interlocked transcriptional feedback loops [3, 4]. Although molecular components contributing to different domains of these circuits have been described, how the loops are connected at the molecular level is not fully understood. Genetic screens previously identified LUX ARRHYTHMO (LUX) [5], also known as PHYTOCLOCK1 (PCL1) [6], an evening-expressed putative transcription factor essential for circadian rhythmicity. We determined the in vitro DNA-binding specificity for LUX by using universal protein binding microarrays; we then demonstrated that LUX directly regulates the expression of *PSEUDO RESPONSE REGULATOR9* (*PRR9*), a major component of the morning transcriptional feedback circuit, through association with the newly discovered DNA binding site. We also show that LUX binds to its own promoter, defining a new negative autoregulatory feedback loop within the core clock. These novel connections between the archetypal loops of the *Arabidopsis* clock represent a significant advance toward defining the molecular dynamics underlying the circadian network in plants and provide the first mechanistic insight into the molecular function of the previously orphan clock factor LUX.

Results and Discussion

LUX Selectively Binds DNA

To determine whether LUX ARRHYTHMO (LUX) can bind to DNA and to identify potential target sequences, we made use of universal protein binding microarrays (PBMs). PBM technology provides a rapid means of comprehensively characterizing the in vitro DNA-binding specificities of transcription factors, regardless of structural class or species of origin [7]. We used custom-designed DNA microarrays consisting of ~44,000 60-mer oligonucleotides that collectively represent all possible 10 bp DNA sequences. Each 8-mer is represented at least 16 times on the array, providing a comprehensive and quantitative in vitro assessment of binding preferences for a given protein. We performed triplicate PBM experiments on two different "all 10-mer" designs, for a total of six replicates. Full-length glutathione S-transferase (GST)-tagged LUX protein was produced in *Escherichia coli*, purified, and applied to the DNA microarray; LUX binding at each DNA spot was detected and quantified using fluorescence-conjugated anti-GST antibody. To determine LUX binding preferences over all 8-mers, we used the 60-mer probe data to calculate enrichment scores (E-scores). E-scores reflect the relative preference of the protein for binding each 8-mer; E > 0.45 is indicative of strongly preferred binding sequences [8]. The highest-ranked 8-mer bound by LUX was AGATACGC (E = 0.487) (Figure 1B; see also Tables S1 and S2 available online). Variations in the first or last position in this 8-mer did not greatly affect the E-score, whereas changes at the third or fourth position drastically decreased it, bringing it to negative values (Figure 1B). The overall LUX binding site (LBS) motif can be represented as GATWCG (where W indicates A or T) (Figure 1A; Table S3). To confirm binding to the identified LBS sequence, we constructed synthetic multimers carrying four copies of the LBS or four copies of mutant versions of the LBS with single or multiple mutations. These multimers were cloned upstream of a yeast minimal promoter::*β-galactosidase* (*lacZ*) transcriptional fusion, and the generated reporters were used in a yeast one-hybrid system to test binding of LUX fused to a GAL4 activation domain (LUX-GAL4AD). LUX bound to the multimerized LBS, but not to the mutated sequences, confirming that LUX selectively binds the sequence GATWCG (Figure 1C). We conclude that LUX is a sequence-specific DNA-binding protein and that we have determined its DNA-binding specificity.

In most systems, distinct transcription factor (TF) families participate in clock networks. Additionally, it is common that several members of a particular family are involved. CLOCK/CYCLE and CLOCK/BMAL1 are basic helix-loop-helix (bHLH) PAS domain TFs that interact to form an activator complex in *Drosophila* [9, 10] and mammals [11], respectively. In *Arabidopsis*, the morning Myb-like TFs CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) homo- and heterodimerize to function as a repressor complex [12, 13]. LUX belongs to a small family of five proteins with a single DNA-binding domain unique to plants, part of the larger group of Myb-like GARP transcription factors [5, 6]. Among these, a protein encoded by a gene on chromosome 5, *At5g59570*, is most similar (72%) to LUX. In particular, their DNA-binding domains share 97% identity (Figure S1A). In addition, its expression pattern, like that of *LUX*, is circadian regulated with a peak in the evening (Figures S1B and S1C); we therefore called this gene *NOX* (from the Latin word for "night"). Because T-DNA insertion lines for *NOX* are not available, we generated RNA interference (RNAi) lines to observe the effects of reduced *NOX* levels. Contrary to *lux* mutants, they displayed robust circadian rhythms (Figures S1D–S1F), suggesting that NOX is not fully redundant with LUX. However,

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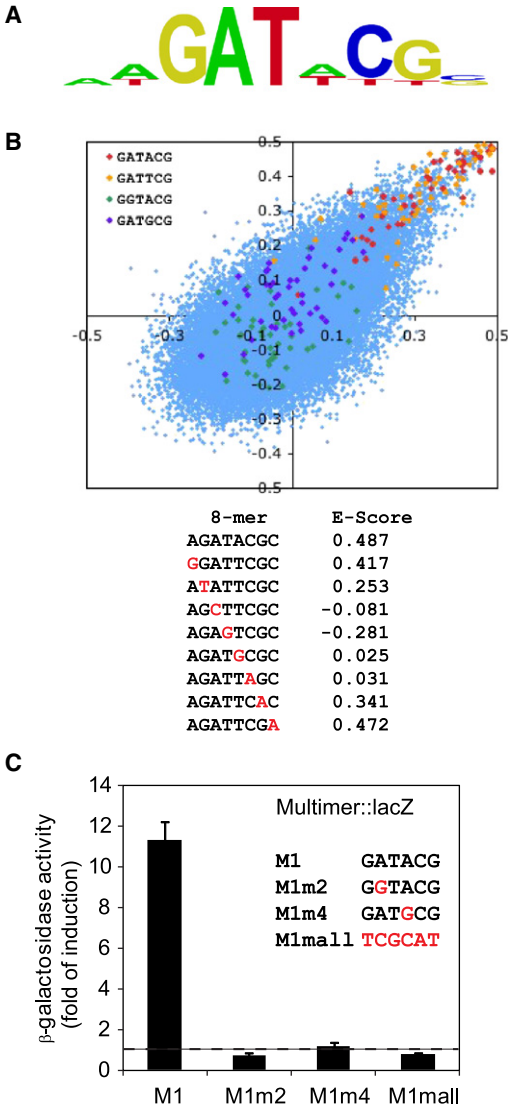

Figure 1. LUX Is a Sequence-Specific DNA-Binding Protein

(A) LUX DNA binding site motif determined by universal protein binding microarray (PBM) experiments.

(B) Effect of point mutations on DNA binding affinity. The scatter plot shows enrichment scores (E-scores) for two "all 10-mer" microarrays of different design; for each design, the E-scores from three replicates were averaged. The E-score correlates with the binding affinity of LUX for the sequence and is measured on a scale of −0.5 (worst) to 0.5 (best). Spots containing the 6-mers GATACG and GATTCG are marked in red and orange, respectively; spots containing the variants GGTACG and GATGCG are marked in green and blue, respectively. Below the scatter plot, E-scores are shown for variants at each position of the most preferred 8-mer AGATACGC.

(C) LUX binding to synthetic multimers of the binding motif in a yeast one-hybrid system. Perfect match or mutant versions of the binding motif were multimerized and cloned upstream of a minimal promoter::*LacZ* transcriptional fusion. Bars represent the fold of induction in β-galactosidase activity in the presence of LUX-GAL4AD over control plasmid (means ± standard error of the mean [SEM], n = 6 independent experiments). The selected mutations were predicted to abolish binding, based on PBM E-Scores.

See also Tables S1–S3 and Figure S1.

NOX was able to bind to the multimerized LBS in the yeast one-hybrid system, but not to multimers of LBS variants (Figure S1D).

TF families are greatly expanded in plants relative to other organisms [14]. However, the DNA-binding specificities for most plant TFs are still unknown because of lack of high-throughput studies like those conducted in yeast or mouse [15, 16]. We showed that LUX is a transcription factor and determined its DNA-binding specificity. The DNA-binding domain in the LUX family is also found in proteins similar to members of two-component signal transduction systems: the B-type *Arabidopsis* response regulators (ARRs), GOLDEN2-LIKE (GLK), and PSEUDO RESPONSE REGULATOR2 (PRR2). It is distantly related to the authentic Myb R1R2R3 repeat originally identified in the mammalian c-Myb oncoprotein [17]. A previous report proposed AGATT, which shares four nucleotides (GATT) with the LBS, as the best target sequence for the B-type ARRs ARR1 and ARR2, based on in vitro studies using their Myb-like motif fused to GST [18]. Subsequently, a study on ARR10 showed through several in vitro techniques that its optimal recognition sequence is also AGATT [19]. The high degree of primary sequence conservation among the DNA binding domains of these GARP proteins and LUX (Figure S1A) is consistent with the ability to bind the same core DNA sequence. Furthermore, 9 of the 11 amino acid residues shown by Hosoda et al. [19] to be critical to the ARR10-DNA interaction are conserved in LUX. The two amino acid substitutions (L187 to V148 and A237 to Y198; Figure S1A) are also present in NOX, which we found to also bind the LBS sequence in yeast one-hybrid assays. It is interesting to note that, although LUX and NOX can bind to the same DNA sequence, they are not functionally redundant, as shown by the arrhythmic phenotype of the single *lux* mutant. The actual overlap between the overall DNA-binding specificities of LUX and NOX has not been investigated yet and might give insight into how these two closely related TFs achieve distinct functions. The functional discrepancy might be due to differences in expression patterns, differences in DNA-binding preferences, or interaction with different proteins that modulate binding activity or transcriptional activity, giving them separate sets of targets. Similarly, REVEILLE1 (RVE1), belonging to the same single Myb-domain subfamily as CCA1 and LHY, is clock regulated with a morning peak and binds to the evening element, like CCA1. RVE1, however, has a distinct function and was shown to be primarily a clock output [20]. Using the universal PBMs, we showed that the LUX binding site motif is longer than the core recognition sequence shared with ARR1, ARR2, and ARR10. Comparing the DNA-binding profile of LUX with other DNA binding sites that might be identified in the future for other family members will significantly help to refine the molecular basis for DNA recognition by the GARP family of TFs, which has over 50 members in *Arabidopsis* [14].

LUX Associates with *PRR9* and *LUX* Promoters In Vivo

We have initially focused on the role of LUX in regulating genes in the central oscillator. The LBS was found in the promoters of several clock genes, including the morning genes *PSEUDO RESPONSE REGULATOR9* (*PRR9*) and *PRR7* and the evening genes *LUX* and *EARLY FLOWERING4* (*ELF4*). In particular, *PRR9* contains a perfect match LBS (GATTCG) 166 bp upstream of the transcriptional start site. We generated strains for yeast one-hybrid, harboring different *PRR9* promoter fragments designed around the LBS (Figure 2A). LUX bound to the fragment containing the LBS, but not to the shorter fragment (consisting of the 5′ untranslated region [UTR] only) lacking this site. Additionally, mutating the LBS abolished binding (Figure 2B), confirming that this sequence is responsible for

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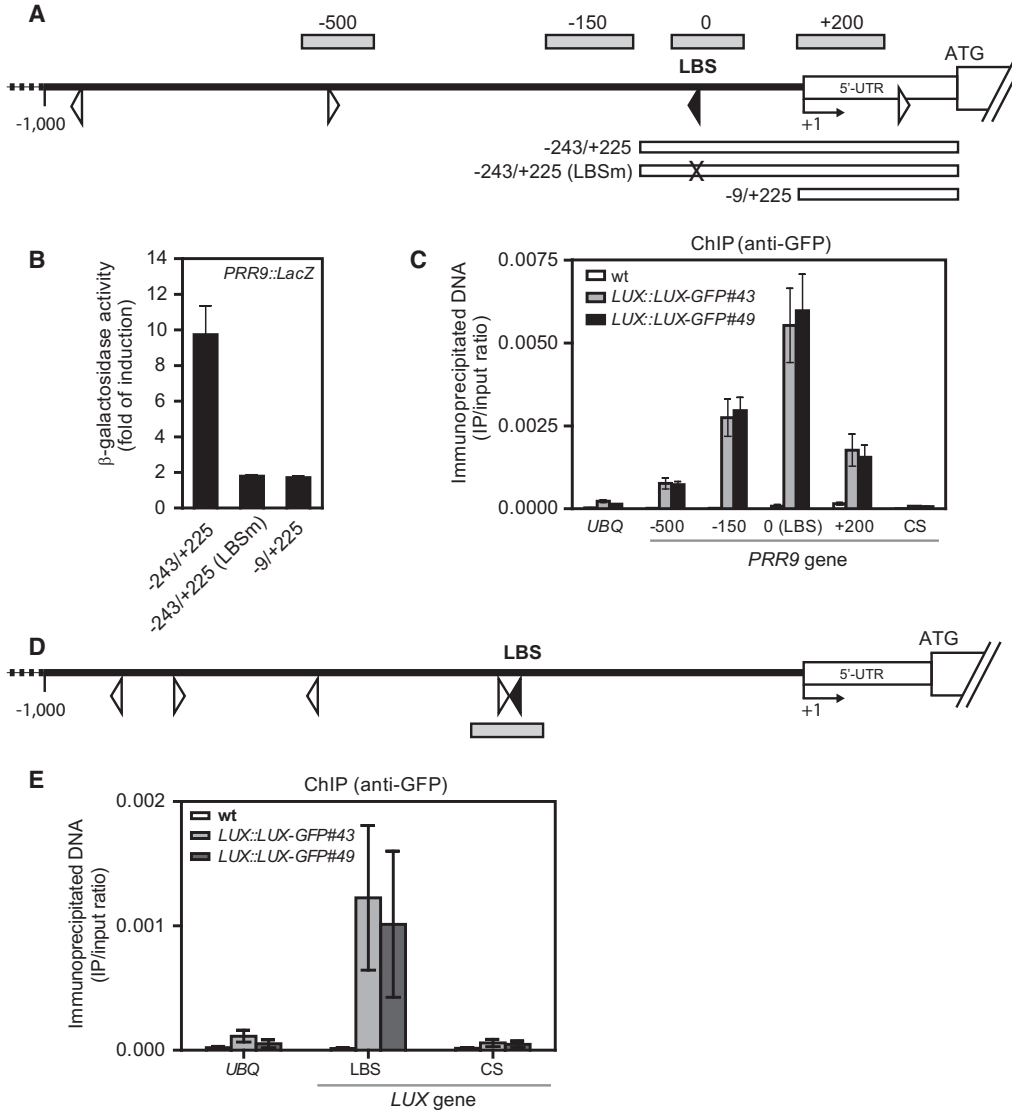

Figure 2. LUX Binds to *PRR9* and *LUX* Promoters In Vivo

(A) Schematic of the *PRR9* promoter (+1 is the transcriptional start site). The black arrowhead indicates the LUX binding site (LBS); white arrowheads indicate degenerate binding sites (GATWKG or GATWCY, where K indicates C or T and Y indicates G or T). White rectangles represent the promoter fragments used for yeast one-hybrid assays in (B), with numbers relative to the transcriptional start site. Grey rectangles show the amplicons used in the ChIP experiments (C) and are numbered using the LBS as a reference (positions relative to the transcriptional start site are detailed in Table S4).

(B) Binding of LUX to *PRR9* promoter in yeast. Bars represent the fold of induction in β-galactosidase activity in the presence of LUX-GAL4AD over control plasmid (n = 4 independent experiments). −243/+225(LBSm) is the −243/+225 fragment with a mutated LBS (GATTCG to TCGGAT).

(C) Binding of LUX to the *PRR9* promoter in vivo. ChIP assays were performed with wild-type *CAB2::LUC* (wt) or *lux-4 LUX::LUX-GFP* (*LUX::LUX-GFP*) seedlings. Plants were grown under 12:12 hr light:dark (LD) cycles and transferred to continuous light (LL). Samples were collected from two independent lines (43 and 49) at Zeitgeber time 14 (ZT14) during the first day in LL and processed for ChIP using an anti-GFP antibody. The immunoprecipitated DNA was quantified using real-time polymerase chain reaction with primers specific for the amplicons represented in (A). The following abbreviations are used: *UBQ*, *UBIQUITIN*; CS, coding sequence. Results were normalized to the input DNA (n = 3 independent experiments).

(D) Schematic of the *LUX* promoter. Black and white arrowheads indicate the LBS and degenerate LBS sequences, respectively, as described in (A). The gray rectangle shows the amplicon centered on the LBS used for ChIP assays.

(E) Binding of LUX to its own promoter in vivo. The ChIP assays were performed as described in (C), with regions of the *UBQ* promoter or *LUX* CS as negative controls.

Values represent means ± SEM in (B), (C), and (E). All primer sequences are detailed in Table S4. See also Figure S2.

specific binding of LUX to the *PRR9* promoter. To investigate LUX binding in vivo, we generated transgenic plants expressing a C-terminal fusion of LUX to GFP under control of either a constitutive promoter (*35S::LUX-YFP*) or the native *LUX* promoter (*LUX::LUX-GFP*) in the *lux-4* mutant background. The *lux-4* allele [5], also characterized as *phytoclock1-1* (*pcl1-1*) [6], carries a nonsense mutation. Thus, in *lux-4* plants, a full-length transcript is generated but only encodes a 149 amino acid protein, truncated at the beginning of the DNA binding domain. Both *lux-4 35S::LUX-YFP* and *lux-4 LUX::LUX-GFP* lines had restored circadian rhythms (Figures S2A and S2B), showing that the LUX-GFP fusion is functional.

Although *LUX* overexpression was reported to cause arrhythmicity after several days in constant conditions [6], our *lux-4 35S::LUX-YFP* lines maintained robust rhythms (Figure S2A). Previously described overexpression lines were in a wild-type background, which might explain the discrepancy with our observations. In the *lux-4 LUX::LUX-GFP* lines, hypocotyl growth was restored (Figure S2C) and *LUX-GFP* transgene expression followed the expression profile of *LUX* in wild-type plants (Figures S2D–S2G), indicating that the LUX-GFP fusion is a good proxy for the native LUX protein. We therefore used these lines for chromatin immunoprecipitation (ChIP) experiments to determine whether LUX is bound to the *PRR9* promoter in vivo. Samples were collected at Zeitgeber time 14 (ZT14) during the first day in continuous light (LL), when LUX protein levels are at a maximum (E.E. Hamilton and S.A.K., personal communication), and were processed for ChIP using an anti-GFP antibody. We analyzed by quantitative PCR (qPCR) different target amplicons from the *PRR9* promoter, as shown in Figure 2A. Amplicons located in the *PRR9* coding sequence and in the promoter of the *UBIQUITIN* (*UBQ*) gene served as negative controls for binding. Several amplicons showed significant enrichment in *lux-4 LUX::LUX-GFP* over wild-type control plants (Figure 2C). Amplicon 0, centered on the LBS, showed the greatest enrichment. Amplicons upstream (−150, −500) and downstream (+200) of the LBS also showed enrichment, with lower values as the distance from the LBS increased. This enrichment is likely due to size variation in DNA fragments produced during sonication (on average 500 bp); as LUX binds to the LBS, adjacent regions are also expected to be pulled down with LUX. As expected, enrichment was not observed for more distal amplicons in the *PRR9* coding sequence or in the *UBQ* promoter. These results show that, as observed in vitro, LUX binds to the *PRR9* promoter in vivo.

Interestingly, although the promoter of *PRR7* has a perfect match LBS and although *PRR7* has a similar expression pattern to *PRR9*, LUX did not bind this promoter region in yeast one-hybrid or ChIP assays (data not shown). *PRR9* and *PRR7* are often considered to be at least partly redundant. For instance, they are wired in the morning loop as negative regulators of *CCA1* and *LHY*, which in turn activate both *PRR9* and *PRR7* expression [21, 22]. *PRR9* and *PRR7* also both participate in temperature entrainment of the clock [23]. However, it has also been shown that *PRR9* and *PRR7* have overlapping but distinct roles in the circadian clock. The single-mutant phenotypes have different light-quality dependencies, whereas an additive phenotype is observed in the double mutant [21, 24]; they display distinct overexpression phenotypes as well [25–27]. Although *PRR9* and *PRR7* have been incorporated as a single component in some mathematical models of the *Arabidopsis* clock [28], they have been separated in others [4, 29]. Additionally, both mathematical modeling [4] and experimental studies [22] indicate that the sequential expression of *PRR9*, *PRR7*, and *PRR5* as a "wave of inhibitors" is required for proper repression of *CCA1* and *LHY* expression from morning until mid-night. Here we provide additional evidence of the distinction between the morning genes *PRR9* and *PRR7* at the level of transcriptional regulation.

Clock oscillator genes in several organisms are known to control their own expression level by negative feedback [30–32]. It has been previously reported that overexpression of *LUX* represses endogenous *LUX* expression and disrupts its circadian expression [6], suggesting that it might be part of an autoregulatory feedback loop. We found that the perfect match LBS present in *LUX* promoter is bound by LUX in vivo, as shown by ChIP assays using the *lux-4 LUX::LUX-GFP* lines (Figures 2D and 2E). The region surrounding the LBS in the promoter was specifically enriched, whereas regions used as negative controls (*UBQ* promoter and *LUX* coding sequence) were not. This result, combined with the observation that *LUX* transcript levels are constitutively high under constant light conditions in the *lux* mutant [6], suggests that LUX defines a new negative autoregulatory feedback circuit within the core clock mechanism. In plants, as in other organisms, self-regulation is a widespread mechanism used to achieve rapid and tight control and is used in cell-cycle regulation (reviewed in [33]) and ethylene signaling (reviewed in [34]). It is also a common feature in clock transcriptional feedback loops, although it appears to be mostly indirect, i.e., involving the activation of a repressor or the repression of an activator rather than direct self-repression. In *Drosophila*, the CLOCK/CYCLE (CLK/CYC) heterodimer binds to E-boxes to activate the key clock genes *PERIOD* (*PER*) and *TIMELESS* (*TIM*). PER and TIM proteins then interact and inhibit CLK/CYC activity [9, 10]. PER-mediated transcriptional repression is associated with the rhythmical binding of PER to circadian promoters, in particular *PER* and *TIM* promoters [35]. In the *Arabidopsis* circadian system, *CCA1* has been proposed to regulate itself based on repression of the endogenous transcript in *CCA1* overexpressing lines [32]. Additionally, the *CCA1* promoter contains a CCA1 binding site [36], a motif shown to be bound by CCA1 in vitro and in vivo [21, 37], although the binding of CCA1 to this element in its own promoter has yet to be confirmed. Similarly, increased expression of *LHY* caused the endogenous gene to lose rhythmic expression, suggesting that *LHY* may also be part of a feedback circuit that regulates its own expression [31]. There is, however, no evidence for this type of self-regulation for *TOC1* [38]. We provide here the first in vivo evidence in *Arabidopsis* of direct self-regulation of a clock transcription factor through binding to its own promoter, indicating that fine tuning of *LUX* levels may be important for proper clock function.

LUX Function Is Lost When Fused to a Strong Activator Domain

Because *PRR9* and *LUX* are antiphasic (Figures 3A and 3B) and LUX binds to the *PRR9* promoter, we reasoned that LUX likely acts as a transcriptional repressor. Therefore, we measured *PRR9* expression in the *lux-4* mutant, starting at the beginning of the night, when *LUX* levels are peaking, and extending through the first day in constant light, because the mutant becomes arrhythmic after release from driven to constant conditions [5]. We found *PRR9* expression to be higher than wild-type levels throughout the entire time course (Figure 3C), consistent with a repressor activity for LUX. To investigate further, we generated transgenic lines overexpressing LUX fused to either the VP64 activation domain [39] (*35S::LUX-VP64*) or the CRES repressor domain [40] (*35S::LUX-CRES*) in the *lux-4* mutant background and monitored circadian rhythms. Interestingly, we found that the *lux-4 35S::LUX-CRES* lines had robust rhythms, showing that the repressor fusion restored rhythmicity (Figures 3D and 3E). On the contrary, expression of the LUX-VP64 activator fusion did not complement the *lux-4* arrhythmic phenotype (Figures 3D and 3F). Moreover, although the *LUX-CRES* construct complemented the hypocotyl growth defect of *lux-4*, the *lux-4 LUX-VP64* lines had an enhanced phenotype with longer hypocotyls than the mutant (Figure 3G).

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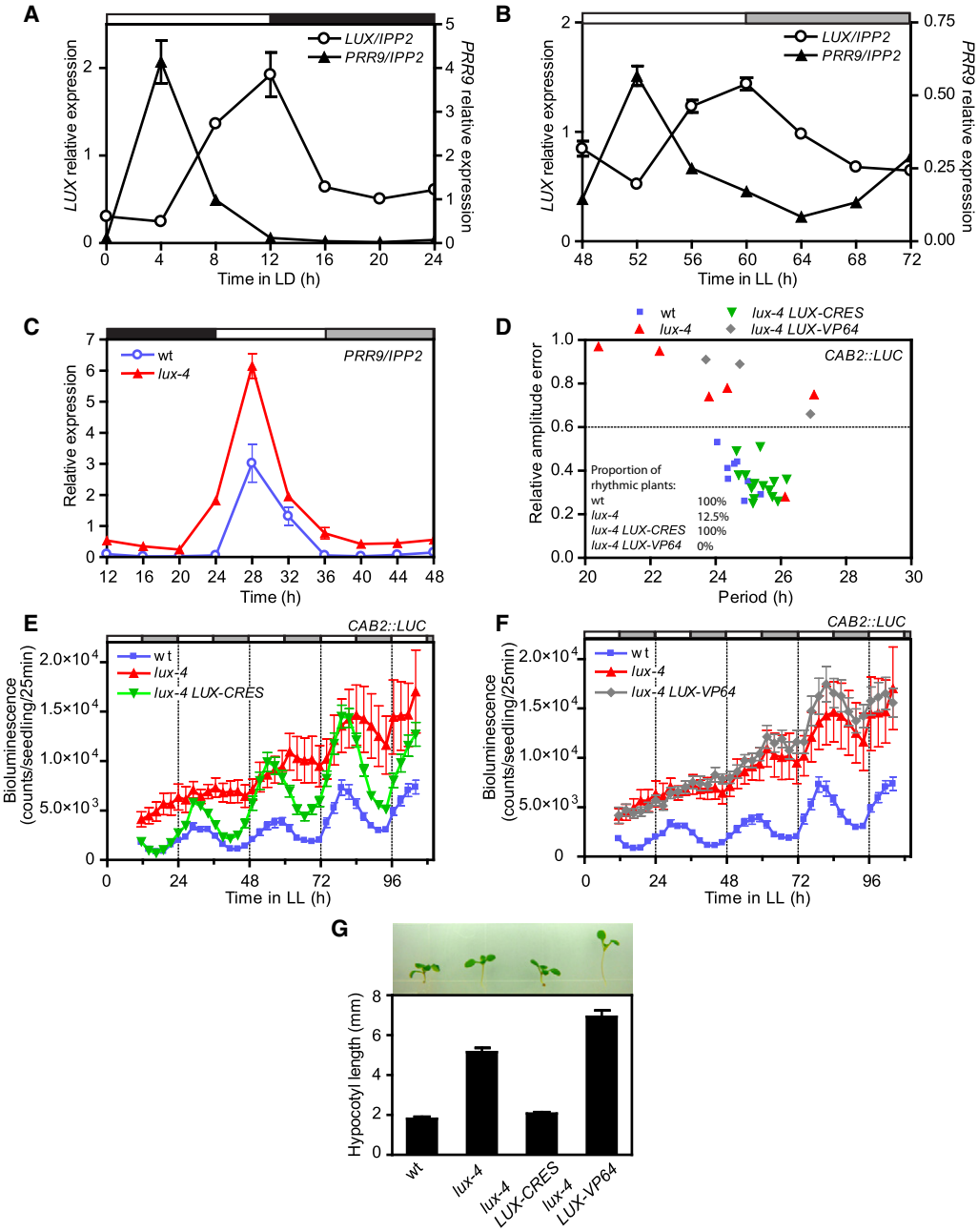

Figure 3. LUX Functions as a Repressor

(A–C) Seedlings were entrained in LD for 10 days before release to LL. mRNA levels were normalized to *IPP2* expression (mean values ± SEM, n = 3 independent experiments).

(A and B) *LUX* and *PRR9* expression in wild-type plants grown in LD (A) or LL (B) cycles.

(C) *PRR9* expression in wild-type *CAB2::LUC* (wt) and *lux-4* mutant in LD released to LL.

(D–G) Effect of the overexpression of LUX fused to either a repression domain (CRES) or an activation domain (VP64) in the *lux-4* mutant. Bioluminescence assays in wild-type *CAB2::LUC* (wt), *lux-4* mutant, *lux-4 35S::LUX-CRES* (*lux-4 LUX-CRES*), and *lux-4 35S::LUX-VP64* (*lux-4 LUX-VP64*) plants (D–F).

(D) Period length and relative amplitude error (RAE) were calculated using fast Fourier transform-nonlinear least-squares analysis. Only plants for which the algorithm retrieves period length and RAE values can be represented on the plot (wt: 8 out of 8; *lux-4*: 6 out of 8; *lux-4 LUX-CRES*: 14 out of 14; *lux-4 LUX-VP64*: 3 out of 16). Individuals with an RAE lower than 0.6 are considered rhythmic.

(E and F) Luciferase activity in wt, *lux-4*, *lux-4 LUX-CRES* (E), and *lux-4 LUX-VP64* (F) lines. Third generation (T3) homozygous plants were entrained in LD for 8 days, then released to LL and imaged every 2.5 hr for 5 days. Values represent means ± SEM (n = 8 for wt and *lux-4*; n = 14 for *lux-4 LUX-CRES*; n = 16 for *lux-4 LUX-VP64*). The experiment was repeated three times with similar results, using two different transgenic lines (data shown for one representative line) selected from an initial screen of 48 primary transformants for each construct (data not shown).

(G) Mean hypocotyl lengths of wt, *lux-4*, *lux-4 LUX-CRES*, and *lux-4 LUX-VP64* plants. Seedlings were grown in LD for 10 days before measuring the hypocotyl lengths (means ± SEM, n = 20 plants).

See also Figure S3.

Measurement of *LUX* expression in these lines showed that they have comparable transcript levels (Figure S3), demonstrating that adding the activator or repressor domain did not prevent expression of the transgenes. Notably, *LUX* endogenous levels in *lux-4 LUX-CRES* lines, but not in *lux-4 LUX-VP64* lines, showed a similar pattern to wild-type, with higher expression in the evening (circadian time [CT] 12 in LL) than in the morning (CT0), consistent with restored rhythms (Figure S3B). Taken together, these results are consistent with LUX acting as a transcriptional repressor in vivo.

Perspectives

Circadian clock networks in most organisms consist of multiple interlocked feedback loops with complex dynamics. Multiple components contribute to each circuit within the overall network. In plants, previous models with morning, evening, and central feedback loops were based mainly on genetic networks derived from mutant analyses, with few mechanistic predictions [31, 32, 41]. However, recent in vivo studies have been contributing more insight into the direct molecular connections within the clock circuit [22, 37]. Our study shows that LUX represses *PRR9* through direct binding to its promoter, adding a novel connection to the previously described circadian network (Figure 4). The *lux* mutation abolishes rhythms entirely in free-running conditions, which cannot be fully explained by the *PRR9* misregulation. Indeed, although *PRR9* overexpressing lines are early flowering like *lux* mutants, they have a short period phenotype [25]. PRR9, like PRR7 and PRR5, associates with the *CCA1* promoter to act as a transcriptional repressor [22]. This could explain the low expression level of *CCA1* in the *lux* mutant [5, 6], where loss of LUX-mediated downregulation of a *CCA1* repressor results in indirect repression. Future studies involving genome-wide identification of LUX direct targets using ChIP-Seq will unravel new elements of critical transcriptional networks that are perturbed in the *lux* mutant, as well as help elucidate output pathways downstream of the clock that are regulated through LUX, making the identification of direct LUX targets other than *PRR9* and *LUX* itself an exciting future challenge.

Few GARP TFs have been characterized in terms of DNA-binding specificity and target genes. Two members of the family, *GLK1* and *GLK2*, have been implicated in the regulation of chloroplast development and were shown to upregulate similar sets of genes primarily involved in photosynthetic function [42]. Attempts at experimentally defining the in vitro GLK1 binding site failed, possibly indicating that GLK1 alone may not bind DNA in a sequence-specific manner. However, a 6 bp motif was overrepresented in the promoters of the proposed target genes. Because GLK1 and GLK2 were previously shown to interact with G-box binding factors in yeast, it has been proposed that GLK proteins act in concert with partners to attain specificity in DNA binding [42]. We are interested in identifying potential partners that modulate LUX molecular function, in particular other clock-regulated proteins that would participate in the same protein complex. Such partners should provide further molecular explanations for the dramatic circadian defect seen in *lux* mutants.

Experimental Procedures

Protein-binding microarrays, yeast one-hybrid, hypocotyl growth assays, luciferase imaging, qPCR assays, and chromatin immunoprecipitation are described in the Supplemental Experimental Procedures.

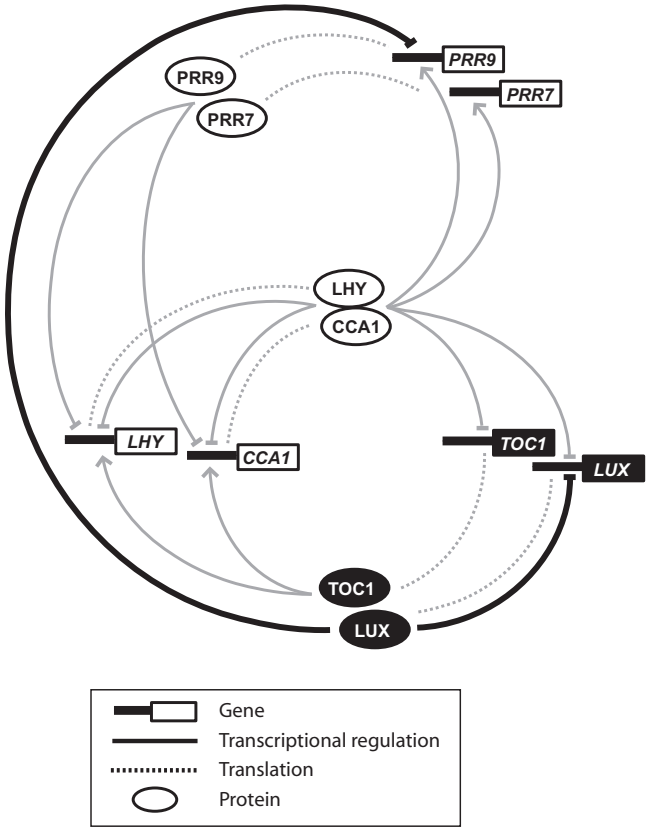

Figure 4. Model for the Proposed Role of LUX in the *Arabidopsis* Clock

LUX is responsible for the downregulation of *PRR9* and *LUX* transcription during late night. Some components of the network were omitted to simplify the model. Morning-expressed genes and proteins are represented in white; evening-expressed genes and proteins are represented in black.

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used. *CAB2::LUC* reporter lines [43] and *lux-4* mutants [5] were previously described.

For *35S::LUX-YFP* lines, we constructed pENTR::LUX by cloning *LUX* coding sequence (forward primer 5′-caccATGGGAGAGGAAGTACAAA-3′, reverse primer 5′-ATTCTCATTTGCGCTTCCACC-3′) in pENTR/D-TOPO (Invitrogen). This construct was recombined using Gateway LR Clonase II (Invitrogen) into pEarleyGate101 [44], generating *35S::LUX-YFP-HA*. For *LUX::LUX-GFP* lines, we cloned a 1870 bp fragment comprising the promoter region up to the previous annotated gene (*At3g46630*), the 5′UTR, and the coding sequence of *LUX* into pENTR/D-TOPO (forward primer 5′-caccCG ACCACAATCAAGGAGTAAT-3′, reverse primer 5′-ATTCTCATTTGCGCTTCC ACC-3′). This construct was recombined using LR Clonase II into pMDC107 [45], generating *LUX::LUX-GFP*. For *35S::LUX-CRES* and *35S::LUX-VP64* lines, pENTR::LUX was recombined into the destination vectors pB7WG2-CRES and pB7WG2-VP64, whose construction is detailed in the Supplemental Experimental Procedures. For *NOX* RNAi lines, an 87 bp fragment of the coding region (forward primer 5′-caccGGCTTATGGTTTATTTTCCC ACT, reverse primer 5′-TCGAAATCATTTTCTATACAAAGGC) was cloned in pENTR/D-TOPO. This fragment is absent from *LUX* and does not show strong homology to any other coding sequence in the genome. The construct was then recombined using LR Clonase II into pB7GWIWG2(II) [46], which allows for overexpression of the hairpin construct targeting *NOX*. All binary constructs were transformed into the appropriate background using *Agrobacterium* (strain GV3101) infiltration [47].

Seeds were gas sterilized and plated on 1× Murashige and Skoog basal salt medium with 1.5% agar and 3% (w/v) sucrose (MS plates). After stratification for 3 days, plates were transferred to a Percival incubator (http://www.percival-scientific.com) set to a constant temperature of 22°C. Light entrainment was 12:12 hr light:dark cycles, with light supplied at 80 μmol m^{-2} sec^{-1}.

Supplemental Information

Supplemental Information includes three figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.12.021.

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