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Integrating ELF4 into the circadian system through combined structural and functional studies

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The circadian clock is a timekeeping mechanism that enables anticipation of daily environmental changes. In the plant Arabidopsis thaliana, the circadian system is a multiloop series of interlocked transcription-translation feedbacks. Several genes have been arranged in these oscillation loops, but the position of the core-clock gene ELF4 in this network was previously undetermined. ELF4 lacks sequence similarity to known domains, and functional homologs have not yet been identified. Here we show that ELF4 is functionally conserved within a subclade of related sequences, and forms an alpha-helical homodimer with a likely electrostatic interface that could be structurally modeled. We support this hypothesis by expression analysis of new elf4 hypomorphic alleles. These weak mutants were found to have expression level phenotypes of both morning and evening clock genes, implicating multiple entry points of ELF4 within the multiloop network. This could be mathematically modeled. Furthermore, morning-expression defects were particular to some elf4 alleles, suggesting predominant ELF4 action just preceding dawn. We provide a new hypothesis about ELF4 in the oscillator—it acts as a homodimer to integrate two arms of the circadian clock. [DOI: 10.2976/1.3218766]
translation feedback loops underlying this mechanism in the model plant Arabidopsis thaliana (Arabidopsis). Progress on entrainment of the oscillator is also emerging (Harmer, 2009). The long-term goal is to fully account for the genetic factors that bridge the transitions of light-perception, which occur at dawn and dusk, and to integrate these features into the oscillator mechanism.

In a recent model of the plant-circadian system, central-clock genes were arranged in three interconnected feedback loops (Locke et al., 2006; Zeilinger et al., 2006). In the core loop, the Myb-like morning transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) repress transcription of the pseudoresponse regulator (PRR) TIMING OF CAB EXPRESSION 1 (TOC1) (Alabadi et al., 2002; Perales and Mas, 2007; Schaffer et al., 1998; Strayer et al., 2000; Wang and Tobin, 1998). Specifically, this morning repression results in a restriction of TOC1 expression, and only as repression is relieved does evening activation occur. Closing the core of the clock, TOC1 is believed to confer a genetic activity that ultimately results in the promotion of CCA1 and LHY transcription at the end of the night (Alabadi et al., 2001, 2002). It was recently established that the TCP transcription factor CCA1 HIKING EXPEDITION (CHE) links the feedback activity of TOC1 to the transcriptional induction of CCA1 (Pruneda-Paz et al., 2009). A morning loop, that includes PRR9 and PRR7, is interlocked with the core loop. CCA1/LHY promotes PRR9 and PRR7 expression, which in return have negative feedback on CCA1/LHY (Farre et al., 2005; Nakamichi et al., 2005a, 2005b). Separately, an evening loop containing GIGANTEA (GI) (Fowler et al., 1999) as a partial constituent of a mathematically defined “Y” component, reciprocally connects to TOC1 expression. The evening-expressed LUX ARRHYTHMO (LUX) encodes a Myb-containing transcription factor that likely functions in a feedback role similar to TOC1 (Hazan et al., 2005; Onai and Ishiura, 2005), and it remains a possibility that LUX could also be a component of the Y activity. In addition, an unknown gene X mathematically is predicted to bridge TOC1 activity in the promotion of CCA1/LHY (Locke et al., 2006; Zeilinger et al., 2006).

Additional genes must be considered in the oscillator mechanism. For example, we have previously hypothesized that dusk expression of EARLY FLOWERING 4 (ELF4) is interlocked with the central CCA1/LHY-TOC1 loop (Kolmós and Davis, 2007; McWatters et al., 2007). ELF4 is necessary and sufficient to promote both CCA1 and LHY and repress TOC1 (McWatters et al., 2007). Importantly, the modularity of the multiloop structure of the circadian system is believed to enable robust and dynamic gene regulation, which is stable under divergent environmental conditions (Herzog, 2007). Positioning genes genetically known to function within the oscillator is thus a critical task.

In the absence of ELF4, the circadian clock fails to sustain rhythmicity under constant conditions and the residual clock rhythms are imprecise (Doyle et al., 2002; Kikis et al., 2005; McWatters et al., 2007). This results in the plants being early flowering, and the elf4 lines have an elongated hypocotyl that is particularly evident after growth under a short-day photoperiod. Both of these phenotypes have been hypothesized to be due to a defect in daylength-sensing (Dowson-Day and Millar, 1999; Doyle et al., 2002; Nozue et al., 2007). Earlier investigations of ELF4 were based on elf4 loss-of-function mutants (Doyle et al., 2002; Khanna et al., 2003; Kikis et al., 2005; McWatters et al., 2007). The first allele was isolated in the Wassilewskija-2 accession (Ws-2, Ws hereafter), termed elf4-1, and it has a deletion of the ELF4 coding region (Doyle et al., 2002). The two Columbia-0 (Col-0) elf4 alleles, elf4-101 and elf4-102, are T-DNA insertion lines that display a null phenotype that is phenotypically less severe compared to elf4-1, regarding amplitude of CCA1 transcript accumulation and hypocotyl length (Khanna et al., 2003; Kikis et al., 2005). In these pioneering studies on elf4, the ELF4-encoded polypeptide remained effectively uncharacterized.

In Arabidopsis, ELF4 belongs to a small gene family and has four sequence homologs (ELF1–ELF4). These sequences lack detectable amino-acid similarity to characterized proteins outside the ELF4 superfamily and, more generally, this family has not been detected outside the plant kingdom (Boxall et al., 2005; Doyle et al., 2002; Khanna et al., 2003). For this reason, sequence repositories have classified the ELF4 family as its own functional class; InterPro DUF1313 (http://www.ebi.ac.uk/interpro/1Entry?ac=IPR009741). A limited comparison of ELF4-like sequences revealed several conserved residues in the ELF4 family (Doyle et al., 2002; Khanna et al., 2003). About 20% of the ELF4 amino-acid residues were shown to be conserved, and two subgroups could be defined. One group contained the Arabidopsis sequences ELF4 and ELF1, and the second group included ELF2, ELF3, and ELF4. Sequences from rice and sorghum were on a branch with ELF2, ELF3, and ELF4 (Khanna et al., 2003), suggesting that a rice ortholog of ELF4 remained to be detected. In contrast, analyses that included McELF4 from iceplant showed that this sequence is on a branch with ELF4, identifying it as an ortholog (Boxall et al., 2005). Transcriptional expression data are consistent with phylogenetic predictions. McELF4 is clock-controlled at the level of transcript accumulation, whereas the rice ELF4-like genes apparently are not (Boxall et al., 2005; Murakami et al., 2007). Thus, the functional conservation of ELF4 is unclear and should be clarified.

In this study, we placed ELF4 within the circadian network using molecular-genetic approaches. We complemented this by informatic approaches to aid in defining ELF4 functionality. Together, these methods enabled us to measure ELF4 conservation and propose the DUF1313 functional domain. Notably, a proposed homodimer and α-helical
structure of ELF4 was empirically confirmed. We then characterized new hypomorphic elf4 alleles that we identified. For this, we took advantage of a targeted screen for Ethyl methanesulfonate (EMS)-mutations (Targeting Induced Local Lesions IN Genomes, TILLING) in ELF4 to generate an allelic series of mutants. In this way, we could systematically dissect the signal network of ELF4 action within the oscillator system. In the elf4 TILLING analysis, we isolated three strong reduced-function alleles that corresponded to residues predicted to be on a surface-charged structure. Additionally, we found that the expression of clock genes was altered throughout the elf4 TILLING collection. Importantly, the subtle elf4 alleles helped the conclusion that ELF4 is important for down-regulation of PRR7 expression during the night. Using this information, we could place ELF4 within the oscillator mechanism, which could be replicated mathematically. From our detailed structural-functional studies, we propose that ELF4 functions as a small dimer critical for buffering perturbations to the evening-arm of the circadian clock. Generally, our work provides a comprehensive approach to the integration of proteins of unknown function within a structural and functional framework of their signaling pathway.

RESULTS

ELF4 is a predicted one-domain protein

The structure of ELF4 is previously undetermined. Accordingly, we used a phylogenetic approach to propose the ELF4 functional domain. ELF4-related sequences from 25 species were identified by basic local alignment search tool (BLAST) in expressed-sequence tagged (EST) cDNA collections and in genomic databases; no ELF4-like sequences were detected outside the plant kingdom. These clones were obtained from various genome projects, and full-length sequences were determined. We used these to generate a multiple alignment (see Supplementary Material, Fig. S1). This revealed a central conserved region between the ELF4/EFL-related polypeptides comprising ELF4-residues 21–93 (the DUF1313 domain), of which a short stretch (residues 58–63) was less conserved. Furthermore, two subgroups could be identified within the alignment: one encompassed an ELF4-related group and the other an EFL-related group. We noted that all predicted polypeptides were ~110–150 amino acids in length, and all were individual domain proteins. Also, outside the central core of conservation, all ELF4-related polypeptides had both amino- and carboxy-terminal extensions. These “tails” were more divergent between the ELF4 and the EFL subgroups. As all ELF4 sequences were most similar within the central part of the protein, this could be the critical domain for structure and function.

There was a high degree of sequence conservation within the ELF4 family (see Supplementary Material, Fig. S1). The ELF4 group was divergent from the EFLs primarily in the most N- and C-terminal regions, and within the ELF4 group, the previously hypothesized ELF4 nuclear localization site (KRRR; residues 8–11) was not conserved (Khanna et al., 2003). Fifteen residues in the central part of ELF4 were fully conserved across the whole family (see Supplementary Material, Fig. S1), a refinement compared to the earlier study (Khanna et al., 2003). Some acidic amino acids (S38, Q48, Q49, and Q55) characterized the ELF4 subgroup followed by a conserved glycine (G74), a serine-lysine pair (sites 77–78), and another serine residue (S84). The conserved residues in the ELF4 family are likely to be important for structural stability and/or for function.

We performed phylogenetic analyses in order to determine the orthology of ELF4 and related sequences in Arabidopsis and other angiosperms. The consensus tree from the Bayesian analysis (Fig. 1) highlights clades that were supported by the data in all the analyses. Notably, one of the well-supported clades contains both ELF4 and EFL1, each of these sequences occurring in a separate clade along with their respective homologs from other eudicot sequences. The other ELF sequences (EFL2–EFL4) represent a recent diversification in a more distantly related gene lineage (Fig. 1). The other clades that receive high support consist of sequences sampled from a single family (grasses, legumes, and tobaccos). There are many sequences from single families or relatively closely related groups that do not form clades in any of the trees. For example, the EFL sequences from the two Solanum and the two Gossypium species are not closely

Figure 1. ELF4 phylogeny. ELF4 Bayesian consensus tree. Majority-rule consensus of 3601 trees (burn-in=402 trees) from two runs of 2 million generations each. Bayesian posterior probabilities indicated support for individual nodes are above the branches as percentages. The ELF4/EFL1 clade is shaded in gray.
related, nor are those from Asteraceae (Helianthus and Lactuca), nor from asterids (Solanum, Helianthus, Lactuca, Hedyotis, and Antirrhinum) and from rosids (Arabidopsis, Citrus, Gossypium, and the legumes). This suggests a complex gene history of duplication and loss. The position of the clade of grass sequences as sister to the ELF4/EFL1 clade is moderately supported in the Bayesian (posterior probability of 0.83) and parsimony (82% bootstrap value; data not shown) trees, and is not supported in the likelihood bootstrap consensus tree (data not shown). As in eudicots, there is evidence of gene duplication in the grasses with, for example, multiple sequences occurring in sugar cane (Fig. 1) and rice (Murakami et al., 2007). Thus, the sequences from grasses appear to be related to the eudicot ELF4/EFL1 lineage, but independent gene diversification in grasses makes it difficult to predict which sequence or sequences might encode ELF4-like function.

ELF4 and EFL structure prediction, ELF4 dimer, and structure refinement: We sought to expand on the structural analysis of the ELF4/EFL family through computational analysis (see Supplementary Material, Fig. S2A). For this we started by inferring the protein folds of the DUF1313 sequences we described above through the direct use of de novo protein structure prediction method ROSETTA. This automated modeling was conceptually similar to the method we used previously (Kolmos et al., 2008). We obtained a consensus prediction from the analysis of all five Arabidopsis and an additional 20 non-Arabidopsis ELF4/EFL proteins. All these proteins were predicted to be α-helical with disordered N- and C-termini, and the sequence-conserved core folds back into a zipper-like confirmation. The nonconserved tails were all predicted to be soluble and to fluctuate between many alternative conformations. Note that the regions of the α-helical fold are exactly the sequence interval conserved in this family (see Fig. 1 and Supplementary Material, Fig. S2A). That ROSETTA consistently generated similar structures was taken as a strong predictor that our modeling represents the energetically stable native protein fold of ELF4/EFL. We thus decided to obtain a more statistically accurate model focusing on ELF4 (from Arabidopsis) through an expanded and integrated use of computational and biochemical methods.

We performed advanced protein structure prediction on ELF4 using the GeneSilico metaserver. All methods tested led to a region, comprising residues 21–93, as forming two α-helices with termini that were intrinsically disordered. This was exactly what we predicted above. This result was further supported by several methods for coiled-coil prediction (see Methods). Finally, de novo structure prediction, with a suite of algorithms, indicated that the central region of ELF4 preferentially folds into a coiled-coil structure (see Supplementary Material, Fig. S2B). Here, as above, the residues 21–57 fold into a helix, the small region 58–63 forms a loop, and 64–93 folds into a second helix (see Supplementary Material, Figs. S1 and S2B). These helices interact with each other. Additionally, the amino- and carboxy-terminal tails extend away from the folded core. We conclude that this is the general monomer fold for all DUF1313 proteins.

To further refine our model, we needed to establish if there was a docking interface in ELF4. To test this, we assessed biochemically if ELF4 could self-associate. First we purified recombinant ELF4 protein and analyzed its migration speed by native polyacrylamide gel electrophoresis (PAGE). As can be seen in Fig. 2(A), ELF4 migrates at ~26 kDa, which is the size of a homodimer. To corroborate the ELF4 homodimer, we assessed the flow-rate of ELF4 on a sizing column. Here, we also found ELF4 to be a tight homodimer [Fig. 2(B)].

To determine if the secondary structure of native ELF4 conformed to the structural predictions, we obtained far-ultraviolet (UV) CD spectra. The averaged spectra revealed a...
strong α-helical signal and disordered signals, and no contributions of β-strands were evident [Fig. 2(C)]. ELF4 was thus found to be in a mixture of helical and disordered folding states.

In accordance with our biochemical findings that ELF4 is an α-helical dimer, we sought to define computationally a probable dimer interface. We performed independent docking analyses for the core region (residues 22–95) with the algorithms GRAMM, DOT, and ZDOCK. The consensus result revealed a symmetrical antiparallel orientation of two monomers. This provided a platform for further model refinement via optimization with ROSETTADOCK. According to the PROQ model quality assessment method, the dimer model (comprising residues 22–95) received a score of 2.610, which provides strong statistical support for the final model. Specifically, MetaMQAP predicted that the dimer core exhibits a root mean square deviation (RMSD) of ~4.2. That the dimerization refinement improved the model is indicated by the “monomer alone” receiving a PROQ score of 1.980 and a MetaMQAP-predicted RMSD of ~4.5. In the final step of model refinement, the intrinsically disordered termini were added with MODELLER (Sali and Blundell, 1993). Our final ELF4 model displayed in Fig. 2(D) and Supplementary Material, Figs. S2C-E represents a plausible structural fold for the ELF4/EFL family.

EFL functional conservation: to determine if the EFL genes are associated with circadian biology, suggestive of a broad conservation of function, we performed EFL expression analyses. In published microarray experiments, the ELF4 expression profile differed from the EFLs (DIURNAL website; see Supplementary Material, Fig. S3A; note, EFL1 was not included as a probe on the ATH1 array). Under continuous light (LL), ELF4 cycled with high amplitude compared to EFL2, EFL3, and EFL4, which were expressed to lower maxima (see Supplementary Material, Fig. S3A). Additionally, the EFL3 profile was neither diurnal nor circadian, and we found that EFL1 expression was not circadian (see Supplementary Material, Fig. S3B). We characterized elf T-DNA insertion lines, but we were unable to detect significant circadian dysfunction in these lines (data not shown). Given the circadian expression of EFL2 and ELF4, together with structural similarity to ELF4 (see Supplementary Material, Fig. S2A), we needed to test the circadian function among EFL sequences.

The EFL expression data indicated that ELF4 circadian function could be conserved in a subset of EFL genes. To test this idea, we evaluated the ability of EFL to ectopically confer circadian rhythmicity in the absence of ELF4. This is plausible as ELF4 is expressed to a higher level than the EFLs (see Supplementary Material, Fig. S3). EFL coding sequences from Arabidopsis and other species were fused to the ELF4 promoter and transformed into elf4-1 plants harboring the CCA1:LUC reporter. Three independent T2 complementation lines were screened under LL, and the results are summarized in Fig. 3 and Supplementary Material, Table S1. In these experiments, we found that EFL1, InEU916969, and HvEU916968 could fully complement the elf4 loss-of-function phenotype. CCA1:LUC was overtly rhythmic, with a similar rescue as that seen with the wild-type control (ELF4p:ELF4). In addition, the expression level of CCA1:LUC was similar to the control for these tested lines (Fig. 3). EFL1, InEU916969, and HvEU916968 also restored elf4 imprecision. The average ratio was about 93% rhythmic seedlings per line in contrast to the average of 70% of seedlings with rhythmicity for elf4-1 (see Supplementary Material, Table S1).

EFL genes from outside the ELF4 group were insufficient to restore ELF4 function. EFL2, EFL3, EFL4, and PtEU916973 all failed to complement elf4-1 phenotypes (see Fig. 3 and Supplementary Material, Table S1). Although many T2 CCA1:LUC lines with ectopic EFL2, EFL3, EFL4, and PtEU916973 expression displayed a modest statistical increase in the ratio of rhythmic seedlings under LL, no transgenic line restored CCA1 amplitude. Taken together, the results from the EFL complementation assays are consistent with our phylogenetic results in that the more distantly related EFL genes (EFL2 to ELF4) do not confer ELF4 activity. In contrast, sequences within the ELF4 group, such as...
**Table I. ELF4 TILLING.** The ELF4 TILLING lines included in this study were named in the order the seeds arrived from the stock center. The site of the nucleotide change is listed according to the position in the genomic sequence.

<table>
<thead>
<tr>
<th>Name</th>
<th>Stock No.</th>
<th>Mutation</th>
<th>Missense</th>
<th>Conservation</th>
<th>Residue change</th>
</tr>
</thead>
<tbody>
<tr>
<td>elf4-215</td>
<td>N87889</td>
<td>G-153A</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>elf4-216</td>
<td>N86936</td>
<td>C-48T</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>elf4-201</td>
<td>N98610</td>
<td>G43A</td>
<td>E15K</td>
<td>Not conserved</td>
<td>Charge change (neg to pos)</td>
</tr>
<tr>
<td>elf4-202</td>
<td>N93293</td>
<td>G52A</td>
<td>E18K</td>
<td>Not conserved</td>
<td>Charge change (neg to pos)</td>
</tr>
<tr>
<td>elf4-207</td>
<td>N90524</td>
<td>C55T</td>
<td>Q19′</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>elf4-208</td>
<td>N87544</td>
<td>C68T</td>
<td>P23L</td>
<td>Not conserved</td>
<td>“Bending” to hydrophobic</td>
</tr>
<tr>
<td>—</td>
<td>N86474</td>
<td>G69A</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>N93422</td>
<td>G72A</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>elf4-209</td>
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<td>G80A</td>
<td>W26′</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>elf4-203</td>
<td>N90093</td>
<td>C93T</td>
<td>R31W</td>
<td>Charge conserved globally (K, R)</td>
<td>Positive to hydrophobic</td>
</tr>
<tr>
<td>elf4-210</td>
<td>N88261</td>
<td>G94A</td>
<td>R31Q</td>
<td>Charge conserved globally (K, R)</td>
<td>Positive to hydrophilic</td>
</tr>
<tr>
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<td>G103A</td>
<td>R34K</td>
<td>Charge conserved in subclade (K, R)</td>
<td>Both positive, size change</td>
</tr>
<tr>
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<td>G133A</td>
<td>R44K</td>
<td>R conserved globally</td>
<td>Both positive, size change</td>
</tr>
<tr>
<td>elf4-211</td>
<td>N86881</td>
<td>C164T</td>
<td>S45L</td>
<td>Hydrophobicity conserved globally</td>
<td>Hydrophilic to hydrophobic</td>
</tr>
<tr>
<td>—</td>
<td>N91818</td>
<td>C164T</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>elf4-212</td>
<td>N86760</td>
<td>C178T</td>
<td>A59V</td>
<td>Not conserved, most are P</td>
<td>Both hydrophobic, size change</td>
</tr>
<tr>
<td>elf4-213</td>
<td>N90652</td>
<td>G222A</td>
<td>G74R</td>
<td>G conserved in subclade</td>
<td>Neutral to positive, size change</td>
</tr>
<tr>
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<td>G340A</td>
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<tr>
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<td>—</td>
<td>N87433</td>
<td>C553T</td>
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</tr>
</tbody>
</table>

**EFL1, InEU916969, and HvEU916968, are sufficient to provide ELF4 activity.**

**ELF4 TILLING**

In order to functionally test our proposed ELF4 structure, we characterized a hypomorphic collection of elf4 mutants that we obtained after a TILLING mutagenesis screen. Using this approach, we isolated ten alleles of ELF4 with missense mutations in the coding region (see Supplementary Material, Fig. S4 and Table I).

Allelic strength: we generated transheterozygous elf4 plants in order to test the allelic strength of the TILLING mutations. For this, we crossed elf4-1 (Ws) harboring a LUC reporter (CCA1:LUC or CCR2:LUC, respectively) as a male to females of the elf4 TILLING lines (Col-0). We note that for all F1 plants, one chromosome of a homologous pair will be entirely Ws and the other entirely Col-0. Thus, all lines were isogenic for genome-wide heterozygocity, except at the locus. We analyzed the F1 plants under LL, and the results are shown in Supplementary Material, Figs. S5A–C.

When the two null alleles elf4-207 and elf4-1 were combined in trans, the CCA1:LUC expression dampened rapidly and the circadian rhythm was lost after ~48 h (see Supplementary Material, Fig. S5A). Furthermore, the mean level of CCA1:LUC was lower in elf4-207×elf4-1 compared to Col-0×elf4-1, and this result confirmed the robustness of the assay. We generated F1 plants with the remaining elf4 TILLING mutations. For plants containing elf4-203 or elf4-212 over the elf4-1 null, we consistently found a short-period phenotype (see Supplementary Material, Fig. S5B and data not shown). Additionally, we found strong allelic strength (short period) for elf4-203 and elf4-212 in F1 plants harboring the CCR2:LUC reporter (see Supplementary Material, Figs. S5E and S5F). We note that loss of LUC rhythmicity (dampening) was only seen in elf4-207×elf4-1 F1 plants (see Supplementary Material, Figs. S5A and S5D). All other allele crosses were similar to the Col-0×elf4-1 results (data not shown), and this could reflect hybrid vigor. The F1 phenotypes thus indicated that elf4-203 and elf4-212 were relatively strong elf4 alleles.

Diurnal gene expression in elf4-207: it was reported that elf4-101 (null mutant in Col-0) had low CCA1 and LHY expression, but unchanged TOC1 levels during first subjective day under continuous red light (Kikis et al., 2005). We sought to expand this clock-expression profiling in the equivalent Col-0 elf4-207 allele, and first analyzed diurnal RNA samples taken from an LD regime. The diurnal phase of several clock genes (CCA1, LHY, PRR9, PRR7, and GI) was largely unchanged in elf4-207, indicating driven rhythms [Figs. 4(A)–4(E)]. This was in agreement with our previous characterization of elf4 loss-of-function in Ws (McWatters et al., 2007). However, we noted that CCA1 and LHY displayed a low amplitude, PRR7 was slightly derepressed in the night, and the GI expression, on average, was higher with lower kurtosis [Figs. 4(A), 4(B), 4(D), and 4(E)]. In addition, we found that the level of TOC1 expression was elevated in...
The expression level is relative to TUBULIN2 (TUB2) and normalized to the average diurnal expression level in Col-0. Gray box indicates night-time for LD profiles, or subjective night or day in LL or DD assays. Gray and black curves represent elf4-207 and Col-0, respectively. The Y-axes represent normalized gene expression and the X-axes are Time (hours).

(A) and (B) Strongly attenuated expression of CCA1 and LHY in elf4-207. (C) PRR9. LL average: elf4-207, 0.60; Col-0, 0.60; P=0.98. DD average: elf4-207, 1.18; Col-0, 0.18; P=1.4×10⁻⁶. (D) PRR7. LL average: elf4-207, 1.97; Col-0, 0.82; P=0.0006. DD average: elf4-207, 1.63; Col-0, 0.95; P=0.002. (E) GI. LL average: elf4-207, 3.21; Col-0, 0.77; P=0.0002. DD average: elf4-207, 1.14; Col-0, 0.34; P=9.5×10⁻⁷. (F) TOC1. LL average: elf4-207, 1.21; Col-0, 0.82; P=0.27. DD average: elf4-207, 0.94; Col-0, 0.50; P=5.6×10⁻⁴. (G) LUX. LL average: elf4-207, 1.16; Col-0, 0.60; P=0.002. DD average: elf4-207, 1.34; Col-0, 0.56; P=8.2×10⁻⁵.

The expression of the evening gene LUX, as this gene was hypothesized to have similar position in the circadian system as TOC1 (Hazan et al., 2005; Onai and Ishiura, 2005). Interestingly, the nighttime expression of LUX was also derepressed [Fig. 4(F)]. Thus, ELF4 genetically functions as a repressor of TOC1 and LUX transcript accumulation in the night phase of a diurnal cycle.

We next assayed the diurnal expression profiles of the ten elf4 TILLING alleles. In these assays, we were unable to detect significant differences for the clock genes tested (data not shown). We conclude here that the LD cycle drives the circadian system in all our elf4 hypomorphic alleles and likely masks any circadian dysfunction.

Circadian gene expression in elf4-207: to expand the expression profiling of elf4-207, we characterized circadian-gene expression under constant conditions, LL and darkness (DD). First, we confirmed that elf4 loss-of-function in Col-0 led to arrhythmicity with low CCA1 and LHY expressions, and a high level of TOC1 under LL, similar to what we previously reported in Ws (Doyle et al., 2002; McWatters et al., 2007). Here, we found that the TOC1 profile had reduced amplitude but was still moderately rhythmic [Fig. 4(F)]. This result differed slightly from the arrhythmicity and high transcript level of TOC1:LUC in elf4-1, and confirmed our earlier observation that an ELF4 null allele in Ws is phenotypically more impaired than a null in Col-0 (McWatters et al., 2007). We then assayed LUX expression in elf4-207 and found a derepression and a shift in the peak phase of expression [Fig. 4(G)]. This result suggested that ELF4 also repressed LUX under LL. For elf4-207, the TOC1 and LUX profiles in DD were similar to the result from the LL time course, except the mean level of both transcripts were relatively higher in DD than under LL [two- and three-fold higher, respectively; Figs. 4(F) and 4(G)]. Thus, ELF4 is required to sustain the circadian transcript accumulation of both TOC1 and LUX without regard to the light environment, but ELF4 has a significant role in the transcriptional repression of these two evening genes in DD.

We finished our elf4-207 characterization by expression analysis of additional central-clock genes under constant conditions. Under LL, two genes in the “morning loop,” PRR9 and PRR7, dampened to peak and mean level of expression, respectively, in elf4-207 [Figs. 4(C) and 4(D)]. Interestingly, this was not simply predictable from the mathematical model, as low CCA1/LHY expression should lead to the absence of PRR9 and PRR7 expressions (Locke et al., 2006; Zeilinger et al., 2006). In DD compared to under LL, the amplitude of the PRR9 and PRR7 expression was dramatically attenuated in Col-0 [most evident for PRR9; Figs. 4(C) and 4(D)]. Overall, we found that the derepression of PRR9 and PRR7 in elf4-207 was more pronounced in DD than under LL. In elf4-207, PRR9 was tenfold, and PRR7 was twofold higher than in Col-0 in DD [Figs. 4(C) and 4(D)]. Regarding GI, under both LL and in DD, we found an increase in the GI expression level in elf4-207 [Fig. 4(E)], although under LL, the peak time was unchanged. We conclude that ELF4 controls the oscillations in the connected morning and evening loops (CCA1/LHY-PRR9/PRR7 and TOC1-Y/GI, respectively) and, thus, ELF4 has a double-entry point into the three-loop oscillator.

Modeling ELF4 as a dual factor in the clock: in order to relate the elf4-207 expression phenotype to a current mathematical model of the circadian system, we modeled a pre-
Interestingly, we found that the elf4-207 qualitatively similar to what we observed experimentally in PRR9 type was mimicked by this simultaneous misexpression of the hypothetical gene as one gene, PRR9, the effect of the elevated clock to repress expression model [Fig. 5(A)]. The expression of had an elevated expression and not in either single misexpression model \[\text{Fig. 5}\]. We note that in this test of the model, the expression \[\text{Fig. 5(B)}\]. Thus, when the three-loop model has simulta-\[\text{Fig. 5}\]. We note that in this test of the model, the expression \[\text{Fig. 5(B)}\]. Thus, when the three-loop model has simulta-

predicted connection point of ELF4 in the clock. As arrhythmic elf4-207 had an unpredictably high PRR9 and PRR7, as well as an unpredictably high GI expression, we modeled the effect of the elevated PRR9/7 expression (here simplified as one gene, PRR9), in addition to the increased expression of the hypothetical gene Y, which is partially explained by GI (Locke et al., 2006). We elevated an arrhythmic PRR9/7 at two-fold compared to the wild type, and also Y at three-fold the wild type, as the experimentally determined transcript levels of PRR9/7 and GI in elf4-207 under LL [Figs. 4(C)–4(E)]. Interestingly, we found that the elf4 null phenotype was mimicked by this simultaneous misexpression of PRR9 and Y using the three-loop model (Locke et al., 2006) (Fig. 5). We note that in this test of the model, the expression of TOC1 was only found to dampen when both PRR9 and Y had an elevated expression and not in either single misexpression model [Fig. 5(A)]. The expression of LHY (that represents both CCA1 and LHY) was rhythmic in the single Y-ox model, whereas it was at background levels in the PRR9-ox single and the double misexpression model [Fig. 5(B)]. Thus, when the three-loop model has simulta-\[\text{Fig. 5}\]. We note that in this test of the model, the expression \[\text{Fig. 5(B)}\]. Thus, when the three-loop model has simulta-

predicted elf4-203 and elf4-212 to have the largest phenotypic changes in the clock-gene expression, based on the transheterozygous test of allelic strength (see Supplementary Material, Fig. S5). Here, we could define elf4-203 to be the strongest hypomorphic elf4 allele in the TILLING panel (Figs. 6 and 7). This allele had a 4–8 h early phase of all genes under LL and most genes in DD, indicative of a short circadian period (Figs. 6 and 7). We note that compared to elf4-207,elf4-203 was found to display wild-type mean expression levels of the morning genes, but an increase in LUX transcript [LUX normalized levels at 2.26 in elf4-203 and
A phase change under LL (Figs. 6 and 7). Similar to elf4-203, elf4-212, elf4-210 had a derepesssion phenotype with respect to the mean level of PRR7. In elf4-210, the average level of PRR7 was elevated in DD compared to elf4-210 under LL (PRR7 normalized levels for elf4-210 under LL at 0.78, and in DD at 1.43; P = 0.008; arrowheads in Fig. 6(D)). Regarding evening-gene expression in elf4-210, thelevel of TOC1 was elevated in DD (TOC1 normalized levels in elf4-210 at 0.96, and Col-0 at 0.50, P = 0.0002), and the LUX expression displayed a phase shift [Figs. 7(B) and 7(C)]. Collectively, from the analysis of the elf4-203, elf4-212, and elf4-210 expression profiles, it appeared that ELF4-mediated control of clock phase and amplitude involves the regulation of LUX levels under LL, and separately the regulation of TOC1, and PRR9 and PRR7 expression levels in DD. This observation is consistent with our hypothesis that ELF4 has a dual entry point to the clock (Fig. 5).

We define elf4-204, elf4-202, and elf4-201 as medium hypomorphic alleles. All three alleles had derepression phenotypes regarding PRR9 and PRR7 expression, where the level was, respectively, the same or higher in DD than under LL (e.g., PRR7 in elf4-204 under LL at 0.40, and in DD at 1.48; P = 0.0002; see Supplementary Material, Fig. S6). In addition, elf4-204 had the same expression level of GI, TOC1, and LUX, respectively, under LL, as in DD (see Supplementary Material, Fig. S6). Both for elf4-201 and for elf4-202, TOC1 levels were high in DD, and LUX was high under LL (see Supplementary Material, Figs. S6B and S6C). Regarding GI, elf4-201 had a high expression and elf4-202 had a low expression under LL (see Supplementary Material, Fig. S6A). We note here that the mean expression levels CCA1 and LHY were normal in elf4-204, elf4-201, and elf4-202 (data not shown). Thus, the phenotypes of three medium hypomorphic alleles indicate that ELF4 can affect the morning and evening loops without a change in the expression levels of CCA1 and LHY.

Our analysis of the four remaining weak alleles, elf4-208, elf4-205, elf4-211, and elf4-213 confirmed a trend of incomplete PRR9 and PRR7 repressions in DD (see Supplementary Material, Fig. S7). In addition, elf4-205, elf4-211, and elf4-213 had similar low expression of GI under LL, as in DD (Fig. 8(E) and data not shown), and similar constant levels of TOC1 were present in elf4-208, elf4-205, and elf4-211 (Fig. 8(E) and data not shown).

Surface model of the ELF4 dimer: we integrated our data from the above experiments into one rational explanation of the structure-function relation of ELF4. This served to connect its phylogeny and positions of sequence conservation with both our elf4 expression phenotypes and the positioning of the amino acids, whose missense replacement result in a phenotype, to the structural model of ELF4. Furthermore, this allowed us to assess the relative orientation of amino ac-
DISCUSSION

Using loss-of-function alleles of elf4, it was previously found that ELF4 is important for clock precision and, in addition, gain-of-function studies supported ELF4 as a repressor of clock periodicity (Doyle et al., 2002; Kikis et al., 2005; McWatters et al., 2007). Here, we analyzed the ELF4 structure-function relationship using a fully integrated and interdisciplinary approach combining phylogenetics, structural modeling, biochemistry, systems modeling, and molecular genetics. These integrated studies first led us to define the ELF4 functional domain and then to predict and biochemically confirm that ELF4 forms a homodimer with α-helical composition. We then extended these analyses by applying a TILLING mutagenesis screen over the ELF4 lo-
curs, where we isolated a new elf4 mutant collection consisting of missense alleles. The mutant phenotypes of the elf4 hypomorphic alleles revealed that ELF4 couples the three loops of the circadian system and, furthermore, that ELF4 connects to the repression of GI, TOC1, and LUX and specifically to dark-repression of PRR9 and PRR7. This supports the placement of ELF4 within two nodes of the oscillating mechanism. Additionally, we note that the hypomorphic alleles confirmed findings that ELF4 is a repressor of the speed of the clock. The structural map of the hypomorphic series allowed us to define the exterior area of the ELF4 dimer critical for function. Interesting, this interface is in a distinct electrostatic surface. Collectively, we conclude from these studies that ELF4 represses morning, as well as evening loops of the circadian system and, in this way, it entrains the clock.

**ELF4 phylogeny**

Our multiple alignment of ELF4 and EFL sequences identified the central/core ELF4 (DUF1313) domain, which was highly conserved. Furthermore, two “tails” could be determined and these, based on the degree of conservation, were predicted to be less important for the ELF4 structure (see Supplementary Material, Fig. S1). Our phylogenetic results identified ELF4 and EFL1 as members of sister gene lineages in eudicots and that the two sequences from *Populus trichocarpa* (VTK45573 and YFS280019) were members of the ELF4 and EFL1 lineages, respectively (Fig. 1). The available sequences from monocots (all from grasses) were more closely related to each other than to either of these genes. Arabidopsis EFL2 to EFL4 formed a clade by themselves and their relationships with ELF4/EFL sequences in other species remains ambiguous. Several species have ELF4/EFL sequences that appear to be distantly related to each other and that also are not closely related to sequences from other species. Together, the evolution of ELF4/EFL genes is characterized by a dynamic history of gene duplication and loss. Further sampling will provide a more comprehensive description of ELF4 versus ELF evolution.

In accordance with our phylogenetic analyses, modeling of ELF4 and many EFL sequences revealed similar predicted structures for these proteins (see Fig. 2 and Supplementary Material, Fig. S2A). We note that the predictions were computationally independent and, thus, the consistency of the data led us to conclude that the general fold of ELF4 had been discovered. All ELF4/EFL sequences were predicted to consist of two α-helices and we therefore tested whether this fold contained ELF4/EFL functional activity. However, by ectopic complementation assays, of expression of ELF genes in the elf4 minus background, we did not find support that the DUF1313 fold was globally conserved for ELF4 function (Fig. 3). We conclude that the difference in ELF4p:EFL complementation activity was consistent with the groupings in the ELF4 phylogenetic tree.

**Missense mutations in elf4**

The sequence conservation in the ELF4 family (see Supplementary Material, Fig. S1) provided a basis for the prediction of the mutant phenotypes of the elf4 TILLING alleles [Table I and Figs. 8(A) and 8(B)]. Specifically, elf4 mutations could be divided into two classes based on the encoded difference in charged residues. Both elf4-201 (E15K) and elf4-202 (E18K) (see Supplementary Material, Fig. S6) encoded a charge change, but as both E15 and E18 were located in the amino-terminal tail (little sequence conservation), elf4-201 and elf4-202 were not predicted to have strong phenotypes. The P23L substitution in elf4-208 (see Supplementary Material, Fig. S7) changed a proline residue that was not conserved, and because this location was at the end of the tail, no major mutation effect was expected. The more centrally positioned mutations were interesting because the central region had the highest number of conserved ELF4-residues and encoded the two α-helical folds (Fig. 2 and Supplementary Material, Fig. S1). In two mutants, elf4-203 (R31W) and elf4-210 (R31Q) (Figs. 6 and 7), the same encoded arginine residue was changed; more importantly the positive charge was globally conserved at this position, hence, the replacement of arginine with an uncharged amino-acid suggested detectable phenotypes in elf4-203 and elf4-210. Both elf4-204 (R34K) and elf4-205 (R44K) (see Supplementary Material, Figs. S6 and S7) encoded no change in charged residues. The sequence conservation in the ELF4 family (see Supplementary Material, Fig. S7) encoded a charge change, but as both E15 and E18 were predicted to have strong phenotypes. The P23L substitution in elf4-208 (see Supplementary Material, Fig. S7) changed a proline residue that was not conserved, and because this location was at the end of the tail, no major mutation effect was expected. The more centrally positioned mutations were interesting because the central region had the highest number of conserved ELF4-residues and encoded the two α-helical folds (Fig. 2 and Supplementary Material, Fig. S1). In two mutants, elf4-203 (R31W) and elf4-210 (R31Q) (Figs. 6 and 7), the same encoded arginine residue was changed; more importantly the positive charge was globally conserved at this position, hence, the replacement of arginine with an uncharged amino-acid suggested detectable phenotypes in elf4-203 and elf4-210. Both elf4-204 (R34K) and elf4-205 (R44K) (see Supplementary Material, Figs. S6 and S7) encoded no change in charged residues. The location of R34 and R44 in the α-helical fold indicated that a size change would confer a phenotype. Additionally in elf4-205, a conserved arginine was replaced with lysine and this coincided with a phenotype. Mainly hydrophobic residues are found at residue 45 in the ELF4 phylogeny, therefore, a phenotypic effect from the replacement of serine with lysine in elf4-211 (see Supplementary Material, Fig. S7) was unexpected. The A59V change in the elf4-212 mutant (Figs. 6 and 7) affected an alanine residue that was not conserved; however, because this alanine was located in the hinge region between the two α-helical folds, an amino-acid size change at this position implied a mutant phenotype. The most C-terminal residue affected in the elf4 collection was G74 in elf4-213 (see Supplementary Material, Fig. S7). This glycine was conserved in the ELF4 subgroup, suggesting a structural role. The replacement of this glycine with arginine introduced a positive charge at this position, in addition to a size change, which was predicted to disorder the ELF4 surface and the α-helical fold. Collectively, the mutations correlated with the expected phenotypic analysis of the mutants when considered in a structural and phylogenetic context.

To confirm the ELF4-specificity of the elf4 TILLING mutations, we analyzed transheterozygous plants (see Supplementary Material, Fig. S5)—a classical genetic tool developed in 1941 (Lewis, 1941)—and an approach used previously in the analysis of TILLING mutants (e.g., Bao et al., 2004; Enns et al., 2005; Mizoi et al., 2006). We used...
elf4-1 (Ws) as the null allele because this is a true null (the ELF4 locus is deleted; Doyle et al., 2002). In this way, using the two reporters, CCA1:LUC and CCR2:LUC, we proved that the short-period phenotypes of the strong elf4-203 and elf4-212 alleles were caused by reduced ELF4 function (see Supplementary Material, Fig. S5). The transheterozygous plants with the other elf4 TILLING alleles did not display phenotypes for LUC-derived bioluminescence rhythms (data not shown), which perhaps indicate heterosis in the hybrid Col-0 × Ws genome.

ELF4 dimer

The coiled-coil-like dimer structure of ELF4 suggested that this fold could serve as an interaction platform. Noting that the ELF4 dimer model has a predicted asymmetric electrostatic surface, this implicated a molecular activity for this protein. The 12 residues conserved in ELF4/EFL1 subgroup were found to be surface-facing and, thus, would be solvent-exposed [Figs. 8(C) and 8(D)]. As these surface residues were conserved, we suggest that they could serve to direct salt-bridge interactions to a protein-interaction target. That residue 31 is positioned within the positive electrostatic cluster could implicate this particular half of the dimer as the surface of such an activity. We found 15 fully conserved residues in the DUF1313 family that appeared within the interior of the dimer structure. This could implicate that these residues are structural for the α-helical fold and for the strong homodimer interaction. Collectively this leads us to hypothesize that ELF4 functions as a molecular “key.” Here, the activity of ELF4 would be to bind a target protein and such binding would activate that receptor molecule whilst assemblage to the ELF4 effector ligand. Future efforts will explore if such a molecular activity exists for ELF4. If so, understanding this activity would move towards a biochemical definition of how ELF4 directs the different arms of the circadian clock.

Clock gene expression in elf4

The elf4 null mutant has dramatically attenuated CCA1 and LHY expression (Doyle et al., 2002; Kikis et al., 2005). More recently, we have shown that TOC1 expression is elevated in the elf4 null (McWatters et al., 2007). In our attempt to extend the network of ELF4 action, we analyzed the expression of clock genes in elf4-207, an elf4 null allele with an apparently identical phenotype to elf4-101 (Khanna et al., 2003). New to this network placement of ELF4, we found that in elf4-207, PRR9 and PRR7 were expressed at a high level relative to the wild type, and this was most obvious in DD [Figs. 4(C) and 4(D)]. Recalling that low CCA1/LHY levels are seen in elf4 (Kolmos and Davis, 2007), we interpret the elevated PRR9 and PRR7 expression as a direct effect of ELF4 because according to the three-loop model the PRR9 and PRR7 genes would be inactivated in the absence of the CCA1 and LHY expression (Locke et al., 2006; Zeilinger et al., 2006). How TOC1 feeds back on CCA1/LHY expression has long been questioned, and this was one reason why a hypothetical gene X was incorporated into mathematical models (Locke et al., 2006; Zeilinger et al., 2006). Until X is identified, its relation to the ELF4 function is an open question. Furthermore, TOC1 has also been placed in a negative feedback with Y/GI. The high levels of both GI and TOC1 expression in elf4-207 [Figs. 4(E) and 4(F)] conform to the notion that GI can only be a partial constituent of Y (e.g., Ito et al., 2008; Locke et al., 2006; Martin-Tryon et al., 2007).

Our comparative analysis of the PRR9 and PRR7 expression in the elf4 collection led us to the conclusion that PRR7 expression in DD is more sensitive to changes in ELF4 activity. Interestingly, this derepression phenotype was also detectable under an LD cycle, where PRR7 was elevated in the night in elf4-207 compared to Col-0 [Fig. 4(D)]. Through conserved cis-regulatory elements CCA1 and LHY are activators of PRR7 transcription (Farre et al., 2005); however, in DD we did not find concomitant derepression phenotypes of CCA1 and LHY in the elf4 alleles. The PRR9 derepression in the strong elf4 alleles was in agreement with earlier findings that PRR9 expression is light-induced and that elf4 gates light input (Ito et al., 2007; McWatters et al., 2007). Collectively, the gated control of PRR9 and PRR7 in DD seemed to have been uncoupled in our elf4 hypomorphic alleles.

LUX is an evening-expressed transcription factor believed to function at the same phase as TOC1 and ELF4 (Hazen et al., 2005; Onai and Ishiura, 2005). We found that the null elf4-207 had elevated LUX expression [Fig. 4(G)]. Under LD cycles, elf4-207 displayed a derepression phenotype for TOC1 and LUX expressions. This difference was also detected in the expression of GI in elf4-207, which could indicate indirect action of elf4 on TOC1 via GI/Y. Furthermore, ELF4 likely has a similar position in a (separate) negative feedback loop with LUX because the lux mutant has increased ELF4 transcript accumulation (Onai and Ishiura, 2005). Therefore, we predict LUX as a candidate for a component of Y, a hypothesis that remains to be tested.

Our analysis of elf4-207 suggests that ELF4 has two inputs, at both PRR9/PRR7 and GI/LUX expression, respectively, and that it functions to repress the (light-induced) expression of these “entry” points [Fig. 8(F)]. This is in agreement with the three-loop model in that a high rate of GI expression would lead to a high TOC1 transcript level. This activity would be in addition to our earlier findings that ELF4 is an entrainment factor (Kolmos and Davis, 2007; McWatters et al., 2007). Our use of the mathematical three-loop model confirms the plausibility of this hypothesis because the simultaneous overexpression of PRR9 and GI resulted in an arrhythmic clock with peak transcript levels of TOC1 and the absence of LHY expression (Figs. 4 and 5).

We appreciate here previous data in support of the above
This could further support our idea that ELF3, one entry point to the clock [Fig. 8(F)]. This dual role could explain ELF4 function in entrainment in that the clock has a different resetting response to dawn and dusk cues (Millar, 2003).

As light is the major entrainment signal (Zeitgeber), the circadian network likely runs differently under LL than in DD. The results of our expression analyses of the elf4 hypomorphic alleles indicate that the circadian system is more compromised in DD than under LL, as phenotypes were more readily detected in DD in the weakest of the elf4 missense mutants. In DD, many core-clock gene transcripts in elf4 are not repressed as they are in the wild type. It is under such conditions that the ELF4 function appears most critical. The mathematical modeling of the circadian system was primarily based on the interconnected-loop model under LD entrainment and with continuous light input (Locke et al., 2006; Zeilinger et al., 2006). Future expansions of the model with ELF4 will help to answer the question about difference in circadian-loop performance under LL vs in DD.

MATERIALS AND METHODS

Nomenclature

In this study, ELF4-LIKE (ELF4-L) for ELF4 homolog, (Khanna et al., 2003) was renamed EFL (for ELF4-LIKE) with the numbering as in Khanna et al. (2003); EFL1 is At2g29950; EFL2 is At1g72630; EFL3 is At2g06255; and EFL4 is At1g17455. The EFL gene name is in accordance with the guidelines for Arabidopsis gene nomenclature (Meinke and Koornneef, 1997).

EST clones

Candidate orthologous sequences were identified using BLAST (Altschul et al., 1990) in GenBank and genome databases (DOE Joint Genome Institute, Physcobase, and TIGR). EST clones were obtained from the respective investigators, and these were fully sequenced. Contigs were assembled using the GCG package (Wisconsin Package, Genetics Computer Group, Madison, WI), and the consensus sequence was determined.

The sequenced EST clones in this study have been deposited in GenBank (http://www.ncbi.nlm.nih.gov): EU919439 (Am), EU916962 (Bv), EU916963 (Cs), EU916964 (Ga), EU916965 (Ga), EU916966 (Ha), EU916967 (Ht), EU916968 (Hv), EU916969 (In), EU916970 (Sl), EU916971 (Ls), EU916972 (Mt), EU916973 (Pt), EU916974 (Sh), EU916975 (So), EU916976 (So), EU916977 (St), EU916978 (Zm), EU916979 (Mp), EU916980 (Gm), and EU916981 (Lc).

The Arabidopsis Genome Initiative locus codes for the genes discussed in this paper are as follows: CCA1, At2g46830; EFL1, At2g29950; EFL2, At1g72630; EFL3, At2g06255; EFL4, At1g17455; ELF4, At2g40080; GI, At1g20620; LHY, At1g01060; LUX, At3g46640; PRR7, At5g02810; PRR9, At2g46790; and TOCI, At5g61380.

Phylogeny

The derived protein sequences of the sequenced ESTs, and additional partial EST sequences from the public databases, were compared using the multiple alignment tool CLUSTALX (Thompson et al., 1994) and processed with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The derived sequences for the predicted ELF4/EFL proteins are intentionally left un-named so that nomenclature projects can define the name relative to the respective plant community.

Phylogenetic analyses of a 204-nucleotide alignment were performed to assess homology of ELF4 and related sequences from angiosperms. Bayesian phylogenetic analyses were performed using MRBAYES 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Two independent analyses were run, where each consisted of 2,000,000 generations with sampling every 1,000 generations. Maximum likelihood bootstrapping was performed using GARLI 0.951 (Zwickl, 2006). Likelihood parameters were estimated on a parsimony tree, using Paup" (Swofford, 2002). Maximum parsimony bootstrap analyses were performed using Paup" (Swofford, 2002) with 1,000 replicates, a heuristic search strategy of ten random sequences addition, tree-bisection-reconnection swapping, and saving multiple most parsimonious trees.

Plant materials

The elf4-1 mutant (Ws) has been described (Doyle et al., 2002). The luciferase lines used were Ws CCA1:LUC and Ws CCR2:LUC (Doyle et al., 2002). T-DNA insertion lines were elfl-2 (Col-0, SALK_135613), elf2-1 (Ws, FLAG_198A02), elf3-1 (Ws, FLAG_140E10), elf3-2 (Col-0, SALK_009170), elf3-3 (Col-0, SALK_092662), elf3-4 (Col-0, SALK_078416), elf4-1 (Col-0, SALK_084137), elf4-2 (Col-0, SALK_058067), and elf4-3 (Col-0, SAIL_837_C07), which were all obtained from NASC. elfl-1 (Ws) and elf4-3 (Ws) were provided from an initial study on EFL genes (Doyle, 2003; Krysan et al., 1999).

TILLING was performed on the entire coding sequence of ELF4, and this region extended 880 bp. The primers used in the TILLING screen were ELF4-fwd: 5’-CCA ACT TCA CAG CTT CAC TCA CG-3’, and ELF4-rev: 5’-TGC AAC AAT CTA ACC ACA AGC CTT CA-3’. In total, 21
new DNA lesions in the ELF4 locus (Table I) were identified. The M3 TILLING plants were backcrossed three times to Col-0 wild type. Cleaved amplified polymorphisms (CAPS)/derived cleaved amplified polymorphic sequences (dCAPS) markers for the TILLING point mutations are listed in the Supplementary Material, Table S2. The homozygous mutant BC3-F2 plants were confirmed by sequencing the affected gene region.

TILLING lines included in this study, with stock-center codes in parentheses: elf4-201 (N89610), elf4-202 (N93293), elf4-203 (N90093), elf4-204 (N91664), elf4-205 (N91652), elf4-206 (N89787), elf4-207 (N90524), elf4-208 (N87544), elf4-209 (N86619), elf4-210 (N88261), elf4-211 (N86861), elf4-212 (N86760), elf4-213 (N90652), elf4-215 (N87889), elf4-216 (N86936), and elf4-217 (N88032). No homozygous F2 plants were found for lines elf4-206 and elf4-217, and for elf4-211 and elf4-212 the segregation pattern differed between seed batches from the same generation suggesting presence of linked lethal mutations in the backcrossed genomes. Transheterozygous (F1) plants were obtained by crossing elf4-1, using pollen from elf4-1 homozygous for the CCA1:LUC or CCR2:LUC reporter genes to the elf4 TILLING lines and Col-0. Multiple F1 seed were used, and F1 data presented represent the analysis of two biological replicates.

**Complementation of elf4-1**

The 35S promoter fragment of the binary vector pJawohl (KanR, gift from Bekir Ulker, MPIZ) was replaced with the ELF4 promoter (amplified with primers 4pf-Ascl, 5′-TAG TAA GGC GCC CCC TCA TGA TTT CCT GCG GTA ATT TCT CTT -3′; and 4pr-ClaI, 5′-TAC CGG ATC GAT AAT AAT TAC CGG ATC GAT AAT TCT CTT -3′) using Clal and Ascl sites to create pJawohl/ELF4p. Subsequent restriction with Ascl and SpeI enabled exchange of the promoter-Gateway cassettes of pJawohl/ELF4p and pLeela (BastaR, gift from Marc Jakoby, MPIZ), to give pJalee4. Arabidopsis ELF4 and ELF genes were amplified from genomic DNA (Ws ecotype). Ipomoea, Hordeum, and Pinus genes were amplified from EST plasmids.

The following primers (with appropriate Gateway overhangs, GW) were used for amplification: attB1-ELF4, 5′-GWF-ATG AAG AGG AAC GCC GAG AGC AAA-3′; attB1-EFL1, 5′-GWF-ATG GAA GCA TCG ATA GAA TCT CGC TGC-3′; attB1-EFL2, 5′-GWF-ATG GAA TCA AGA ATG GAA GGA GAT-3′; attB1-EFL3, 5′-GWF-ATG GAG GGA GAC ACA ATA CCT TCT GCT GCT-3′; attB1-HvEU916968, 5′-GWF-ATG GAG AAC AGC AGC GCC CGG GAG-3′; attB1-InEU916969, 5′-GWF-ATG GAG AAC TCA CGA GCC GTA-3′; attB1-PtEU916973, 5′-GWF-ATG GAG GCC GAA GCA TAT TCT GCT-3′; atB2-EFL1, 5′-GWR-AGA ACC GGT GTT GGT AGT TGT TGT GGT G-3′; atB2-EFL2, 5′-GWR-CCC GGA TCT AAA TCT CTG G-3′; atB2-EFL3, 5′-GWR-ATT AAG CAG GCC TTA TTC TCT T-3′; attB2-EFL4, 5′-GWR-ACC GGA TAA TCT CTT CTC G-3′; attB2-ELF4, 5′-GWR-AGC TCT AGT TCC GGC AGC ACC A-3′; attB2-HvEU916968, 5′-GWR-CTG TGT GGG GCG CCG CCT CTT-3′; attB2-InEU916969, 5′-GWR-CTG TTC CCG GCC TCC GGA GCG-3′; and attB2-PtEU916973, 5′-GWR-AAA CTG AGG TCG AAA TCT CTT-3′.

The amplified fragments were inserted into the Gateway pDONR207 vector and then shuttled into pJawohl/ELF4p to complete construction design. The resultant plasmids were then introduced to Agrobacterium tumefaciens GV3101 pMPORK. The cloned pJalee4/ELF constructs were confirmed by sequencing and transformed into elf4-1 plants harboring CCA1:LUC by a modified floral-dip method (Davis et al., 2009). Three independent T2 lines for each T-DNA, which behaved similarly in at least two experiments, were included in this report.

**Structure prediction**

Automated ROSETTA predictions were exactly as described (Kolmos et al., 2008). Advanced protein structure prediction, including secondary structure, disorder, and tertiary fold, was performed using GeneSilico metaserver (https://genesilico.pl/meta2) (Kuworski and Bujnicki, 2003). Prediction of potential coiled-coil regions was performed using several tools including COILS, MULTICOIL, MARCOIL, and PAIRCOIL2 (Delorenzi and Speed, 2002; Lupas et al., 1991; McDonnell et al., 2006; Wolf et al., 1997). The three-dimensional structure modeling of the ELF4 monomer was carried out using a local installation of ROSETTA (Simons et al., 1997). We generated 50,000 decoys and clustered them with lower bound for size of the top cluster set on 25, minimal clustering threshold 2, and maximal clustering threshold 5. A lowest-energy conformation from the largest cluster was considered as the most likely structure.

Modeling of the dimeric structure of ELF4 was done for the helical region of the monomer core with GRAMM, DOT, and ZDOCK with default parameters (Chen and Weng, 2002; Mandell et al., 2001; Vakser, 1996). The 30 best-scoring decoys from each method were collected and clustered using MAXCLUSTER (www.sbg.bio.ic.ac.uk/~maxcluster/index.html). Dimer optimization was done with ROSETTADOCK (Schuler-Furman et al., 2005), starting from the orientation corresponding to the largest cluster. The quality of models was assessed using PROQ and METAMQAP (Pawlowski et al., 2008; Wallner and Elofsson, 2003).

**ELF4 protein**

The ELF4 coding region was inserted into pET-28a+ vector (Novagen) using NdeI and Xhol cloning sites. Recombinant N-terminally His-tagged ELF4 was produced in BL21 (DE3)
E. coli strain (Novagen) and purified using HIS-Select nickel affinity gel (Sigma). The native-PAGE analysis was performed using 15% acidic native-polyacrylamide gel. Bands were photographed after Coomassie blue staining. The calibration standards used in the native-PAGE and gel filtration analysis were as follows: albumin, 67 kDa; chymotrypsigenin A, 25 kDa; ovalbumin, 43 kDa; and ribonuclease A, 13.7 kDa. Molecular mass estimation over gel filtration was performed to confirm the molecular mass of the purified ELF4 protein. For this, a Superdex 200 PC 3.2/30 (Amersham Biosciences) column was used with the flow rate 50 μl/min. Buffer: 250 mM NaCl, 50 mM sodium-phosphate buffer pH 8, and 10 mM β-mercaptoethanol.

Circular dichroism
Far-UV circular dichroism measurements were obtained using a JASCO model J-715 (JASCO, Gross-Umstadt, Germany). All measurements were performed at room temperature in a 1 mm path-length cuvette. For each measurement, 20 spectra were accumulated at a scan speed of 50 nm/min with a step resolution of 0.1 nm. The spectra were corrected for a protein-free spectrum obtained under identical conditions. Noise reduction was applied according to the JASCO software. Protein samples were at 0.14 and 0.22 mg/ml in 5 mM potassium-phosphate buffer pH 7.5. The spectra were normalized and the average was calculated. Secondary structure prediction was carried out using SELCON3 (Sreerama et al., 1999; Whitmore and Wallace, 2008).

Growth conditions and luciferase imaging
Seeds were surface sterilized and plated on 2.2 g/l Murashige and Skoog (1× MS) medium (4.4 g/l; adjusted pH to 5.7) with 3% sucrose and 1% agar before being stratified at 4°C for 2–3 days and transferred to 12L:12D growth cabinets with 3% sucrose and 1% agar before being stratified at 4°C.

RNA profiling
Total RNA was isolated with RNeasy Plant Mini Kit (Qiagen) from replicate samples of 1-week-old seedlings entrained in 12L:12D or those that were subsequently released into LL or DD. Total RNA (2 µg) was treated with DNase I (Fermentas) and reverse-transcribed using oligo(dT)15 primer (Roche) and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturers’ instructions. qPCR was performed on 2 µl 1:10 diluted cDNA in a 20 µl reaction with IQ SYBR Green Supermix (Bio-Rad). All qPCRs were performed in the iCycler iQ5 Multicolor Real-time PCR Detection System (Bio-Rad). Each run included a standard curve, which was generated from serial dilutions of a pool of cDNA samples and a melting curve, which ensured amplification of one specific gene product. The amount of template in unknown samples was calculated from the threshold value by the iCycler software using the standard curve results. Measured transcript levels were normalized to the reference gene (TUB2) and subsequently also to the average diurnal level for the respective gene in wild type. The presented results are representative of a two entirely independent biological replicates, both of which were separately assessed in triplicate. Error bars represent adjusted standard deviation, calculated based on the pooled standard deviation

Rhythm analysis
Luminescence levels were quantified and analyzed essentially as described (McWatters et al., 2000; Thain et al., 2000), using the macrosuites TopTempII and Biological Rhythms Analysis Software System (BRASS) (Southern and Millar, 2005) (both available at http://millar.bio.ed.ac.uk), and fast Fourier transform nonlinear least squares (Plautz et al., 1997). Sustainability (precision) of rhythms was derived from measurements of the RAE (Allen et al., 2006; Izumo et al., 2006).
pression were initial level of mRNA, 0.0603 nM; constitutive expression level, 0.3 nM/h; and constitutive translational rate constant, 0.2485 1/h.

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REFERENCES


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