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Formation of an SCF^{ZTL} complex is required for proper regulation of circadian timing

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Summary

The circadian timing system involves an autoregulatory transcription/translation feedback loop that incorporates a diverse array of factors to maintain a 24-h periodicity. In *Arabidopsis* a novel F-box protein, ZEITLUPE (ZTL), plays an important role in the control of the free-running period of the circadian clock. As a class, F-box proteins are well-established components of the Skp/Cullin/F-box (SCF) class of E3 ubiquitin ligases that link the target substrates to the core ubiquitinating activity of the ligase complex via direct association with the Skp protein. Here we identify and characterize the SCF^{ZTL} complex in detail. Yeast two-hybrid tests demonstrate the sufficiency and necessity of the F-box domain for *Arabidopsis* Skp-like protein (ASK) interactions and the dispensability of the unique N-terminal LOV domain in this association. Co-immunoprecipitation of full-length (FL) ZTL with the three known core components of SCF complexes (ASK1, AtCUL1 and AtRBX1) demonstrates that ZTL can assemble into an SCF complex *in vivo*. F-box-containing truncated versions of ZTL (LOV-F and F-kelch) can complex with SCF components *in vivo*, whereas stably expressed LOV or kelch domains alone cannot. Stable expression of F-box-mutated FL ZTL eliminates the shortened period caused by mild ZTL overexpression and also abolishes ASK1 interaction *in vivo*. Reduced levels of the core SCF component AtRBX1 phenocopy the long period phenotype of *ztl* loss-of-function mutations, demonstrating the functional significance of the SCF^{ZTL} complex. Taken together, our data establish SCF^{ZTL} as an essential SCF class E3 ligase controlling circadian period in plants.

Keywords: E3 ligase, F-box, circadian clock, ZEITLUPE, SCF, *Arabidopsis*.

Introduction

The circadian timing system provides for the appropriate phasing of numerous physiological and biochemical processes. Photosynthesis, stomatal opening, hypocotyl elongation and photoperiodic flowering are representative of the wide range of growth and developmental phenomena controlled by the circadian clock (Barak *et al.*, 2000; McClung, 2001). The circadian clock comprises three major components: an input pathway, a central oscillator and an output pathway. The input pathway senses and transduces environmental cues to the central oscillator which generates rhythmicity through the coordinate workings of one or more transcription/translation feedback loops (Young and Kay, 2001). Oscillator activity is then manifested as overt rhythm through one or more output pathways. Apart from the oscillations of mRNA and protein abundance, protein phosphorylation and degradation are additionally involved in maintaining the 24 h cycle (Kim *et al.*, 2003; Lee *et al.*, 2001).

In plants, several classes of proteins have been identified which alter the robustness or period of the circadian system when mutated or mis-expressed. Of these, two gene families have emerged as likely key components of the central oscillator. The *PSEUDORESPONSE REGULATOR* (*PRR*) gene family combines elements of the response regulator proteins involved in bacterial two component signal transduction with a conserved domain common to the CONSTANS plant transcription factor family. Misexpression of any one of the five family members disrupts normal circadian function (Ito *et al.*, 2003; Makino *et al.*, 2002; Matsushika *et al.*, 2000, 2002; Michael *et al.*, 2003; Sato *et al.*, 2002; Somers, 2001; Strayer *et al.*, 2000; Yamamoto *et al.*, 2003). At least one member of this family, *TOC1* (*PRR1*) is negatively

regulated by the second essential element of the central oscillator, the CCA1/LHY class of single Myb-domain containing transcription factors (Alabadi *et al.*, 2002; Carre and Kim, 2002; Mizoguchi *et al.*, 2002; Wang and Tobin, 1998). This finding, together with evidence that *TOC1* positively regulates *CCA1/LHY*, has formed the basis of a rudimentary feedback loop in plants (Alabadi *et al.*, 2001). One member of a third gene family, *ZEITLUPE* (*ZTL*), has been linked molecularly to these two (Mas *et al.*, 2003).

ZEITLUPE encodes a 66-kDa protein containing three distinct domains, a PAS-like LOV domain near the N-terminus, and an F-box and six kelch repeats comprising the remainder of the protein (Somers, 2001; Somers *et al.*, 2000). The ZTL LOV domain is highly similar to the flavin-binding region of the blue light photoreceptors NPH1 (*Arabidopsis*) and WHITE COLLAR 1 (WC-1; *Neurospora*), and binds flavin (FMN) *in vitro* (Cheng *et al.*, 2003; Imaizumi *et al.*, 2003). The F-box is an indispensable element of a broad range of metazoan F-box proteins which interact with SKP1, CULLIN, and RBX1 to form an Skp/Cullin/F-box (SCF) complex that recruits specific substrates for ubiquitination and subsequent proteolysis by the 26s proteasome (Deshaies, 1999; Vierstra, 2003). Protein–protein interaction domains such as leucine-rich repeats and WD-40 repeats, which lie C-terminal to the F-box, determine the specificity of the F-box protein. In ZTL, this region is composed of six kelch repeats.

Recent studies have shown that F-box proteins play an important regulatory role in a wide variety of developmental and physiological responses (Vierstra, 2003 and references therein). The presence of nearly 700 F-box proteins in *Arabidopsis*, together with 33 SKP (ASK in *Arabidopsis*), cullin and RBX genes makes possible the formation of a large number of SCF complexes (Gagne *et al.*, 2002; Vierstra, 2003). This combinatorial potential suggests a multitude of SCF complexes may exist to target a wide range of substrates at different times or locations, thereby conferring a fine degree of regulation over diverse cellular processes.

The unique combination of three distinct domains of ZTL suggests that it may mediate a light-regulated protein degradation of critical clock components, providing a link between the circadian phototransduction pathway and the central oscillator. A recent report has implicated TOC1 as at least one of the substrates of an SCF^{ZTL} complex (Mas *et al.*, 2003), but definitive evidence for ZTL association with *bona fide* SCF components has been lacking. Here we demonstrate that ZTL associates with all known core SCF complex components *in vivo*. We define the F-box as the indispensable motif for this association and show the functional requirement of core SCF components for proper circadian function. We also establish that a functional F-box is required for the normal proteasome-dependent degradation of ZTL itself.

Results

ZTL interaction with ASK1 requires a functional F-box

An *Arabidopsis* two-hybrid library (Clontech; 5.8×10^5 transformants) was screened using full-length (FL) LexA-ZTL. Sixty-three colonies were recovered on selection and showed β-galactosidase activity. Further restriction analysis of polymerase chain reaction (PCR)-generated amplicons of the recovered plasmids identified three classes of digestion profiles. The most abundant class was sequenced and was identified as the FL *SKP1* homolog *ASK1* (At1g75950). This finding confirmed a previous report of targeted testing of FL ZTL with a variety of ASK cDNAs in similar yeast two-hybrid assays (Risseeuw *et al.*, 2003). We created a series of deletion constructs and point mutations in ZTL to test which domains are required for ASK interaction. The F-box and kelch domains together (F-KELCH; residues 191–609; Figure 1, construct 2) were sufficient to mediate a productive interaction with ASK1, indicating that the amino terminus of the protein (residues 1–190), which contains the LOV domain, is unnecessary for this interaction (Figure 1). Previous reports have suggested that the LOV domain may confer a light-dependent activity on ZTL (Cheng *et al.*, 2003; Imaizumi *et al.*, 2003; Somers *et al.*, 2000), but in two-hybrid assays we observed no apparent differences between FL ZTL-ASK1 interactions in the light and dark in yeast (data not shown).

We next determined the requirement of the F-box for ASK1 interaction. In the context of the F-kelch construct we mutated two highly conserved amino acids in the F-box region (Schulman *et al.*, 2000; L200 to A and L213 to A; Figure 1, construct 3) or deleted the F-box region entirely (residues 248–609; Figure 1, construct 4). Both changes eliminated interaction with ASK1 in yeast (Figure 1).

The high degree of conservation among SCF components between yeast and plants (Risseeuw *et al.*, 2003) allows that interaction between ZTL and ASK1 might be facilitated by yeast homologs in the two-hybrid assay. We performed *in vitro* interaction tests to determine whether the two proteins alone were entirely sufficient for their association. FL and deletion versions of GST-ZTL fusion proteins were expressed in *Escherichia coli* and immobilized to glutathione–agarose beads (Figure 2a). pASK1 was *in vitro* transcribed and translated in the presence of S^{35}–methionine and incubated with the GST-ZTL-bound resin. GST-FL ZTL protein and GST-F-kelch protein bound ASK1 well, whereas GST-LOV protein showed no binding even when in excess relative to the other forms (Figure 2a,b). The two L-to-A mutations in the F-box (GST-Fmut) reduced ASK binding to nearly undetectable levels (Figure 2b). These results confirm the yeast two-hybrid data and demonstrate the necessity of the F-box for ZTL-ASK1 interaction *in vitro*.

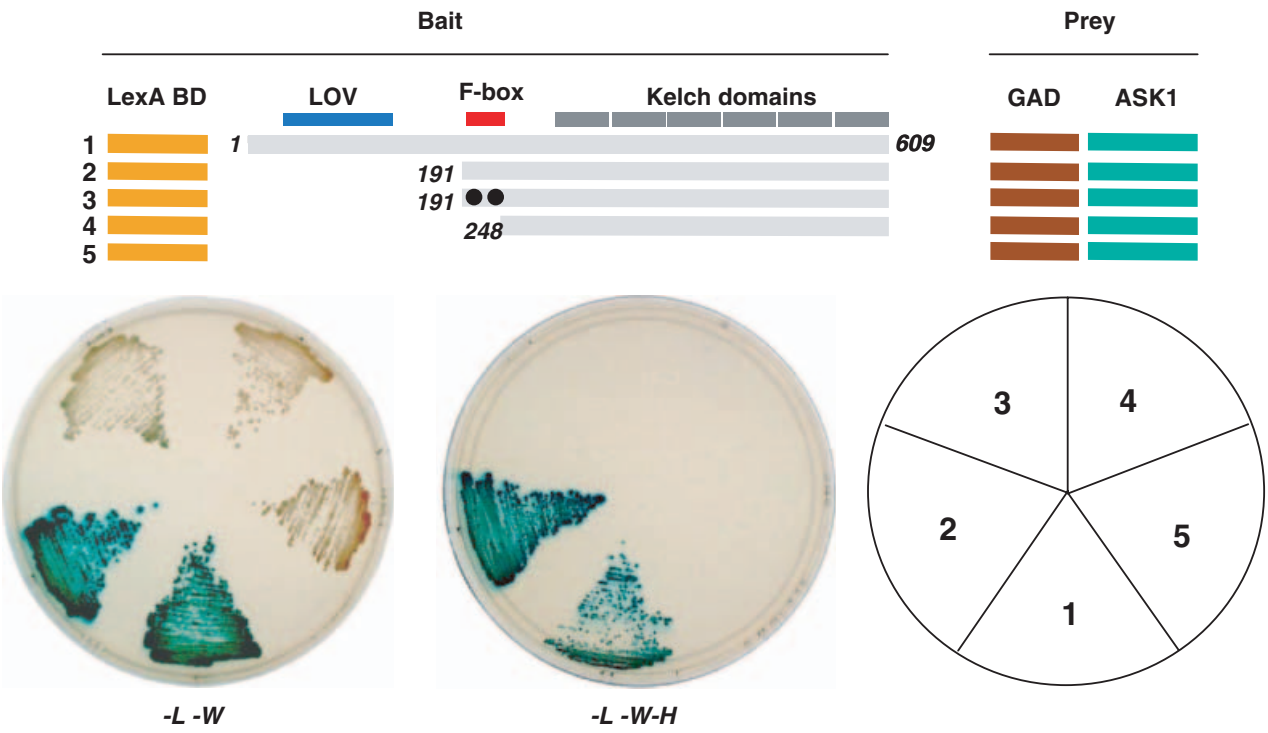

Figure 1. ZEITLUPE (ZTL) interaction with ASK1 in two-hybrid assays requires the F-box.
The LexA DNA binding domain (BD) was fused to full-length ZTL and different deletion versions of ZTL (see text) and tested for interactions with ASK1 fused to the GAL4 activation domain (GAD) in yeast two-hybrid assays. Activation of the LexA-LacZ reporter (blue) and LexA-His selectable markers were used as indicators of productive interactions. Black dots indicate replacement of two conserved F-box consensus amino acids with functionally non-conserved residues. -L, absent leucine; -W, absent tryptophan; -H, absent histidine.

ZTL assembles into an SCF complex in planta

In *Arabidopsis*, the F-box proteins TIR1, COI1 and UFO have been well characterized and shown to assemble into SCF complex in association with ASK1 and AtCUL1 (Gray *et al.*, 1999; Wang *et al.*, 2003; Xu *et al.*, 2002). We further investigated whether ZTL can assemble into a functional SCF complex *in planta*. Green fluorescent protein (GFP) was fused as an N-terminal (35S:GFP:ZTL) tag to the FL ZTL cDNA under the control of CaMV-35S promoter and stably introduced into *Arabidopsis*. Two independent transgenic lines with differing expression levels of fusion protein (GFP-ZTL low; GFP-ZTL high) were chosen for further analysis (Figure 3). Using an α-GFP polyclonal antibody, immunoprecipitates were obtained from clarified protein extracts of plants expressing both levels of the fusion protein, and these were subsequently immunoblotted and probed with ZTL-specific α-ZTL antibody (Kim *et al.*, 2003). Appropriately sized protein bands in both extracts migrate at the identical position of the GFP-ZTL band from immunoblotted plant extracts (Figure 3; lanes 2, 3, 5 and 6). The GFP-ZTL extracts were also immunoprecipitated and immunoblotted with α-ZTL antibody, identifying a band of GFP-ZTL fusion protein size and also the native ZTL protein (Figure 3; lane 8).

Three characteristic components of known SCF complexes are *Arabidopsis* Skp-like proteins (ASKs), cullin and RBX1 (Devoto *et al.*, 2002; Gray *et al.*, 1999; Hellmann *et al.*, 2003; Xu *et al.*, 2002). We tested the α-GFP immunoprecipitates for the presence of these components using antibodies raised against ASK1, cullin and human RBX1. The results from both GFP-ZTL-expressing lines show that ASK1, AtCUL1 and AtRBX1 stoichiometrically co-immunoprecipitate with GFP-ZTL (Figure 3; lanes 5 and 6, all panels). The strong doublet observed with the α-ASK1 antibody is consistent with the initial report that this antiserum recognizes both ASK1 and ASK2 (Gray *et al.*, 1999), and indicates that both proteins interact with ZTL. We observed similar results with plants expressing a C-terminally fused GFP tag (35S:ZTL::GFP) (data not shown), and when α-ZTL antibody was used for immunoprecipitation (Figure 3; lane 8, all panels). The 35S::GFP transgenic plants showed no ASK, cullin or RBX1 immunoreactive bands, demonstrating the specificity of the associations for ZTL (Figure 3; lane 4, all panels).

ZTL does not dimerize in planta

PAS/LOV domains of circadian clock proteins in other eukaryotes mediate protein–protein interactions (Ballario *et al.*,

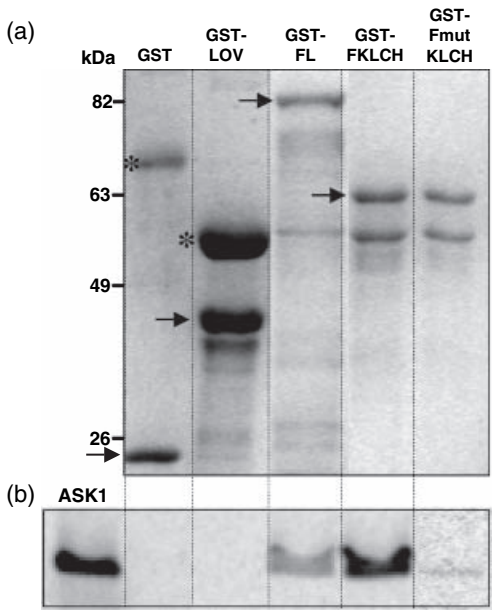

Figure 2. The F-box is necessary for ASK1-ZTL interaction *in vitro*.
In vitro synthesized S^{35}-labeled ASK1 was incubated with GST- ZTL full length (GST-FL) or GST- ZTL deletion forms immobilized to glutathione–agarose beads as indicated.
(a) Coomassie-stained gel of GST-fused proteins used for the *in vitro* binding assay. All GST-fusion proteins were produced in *Escherichia coli* and immobilized to glutathione–agarose beads. Arrows indicate positions of respective fusion proteins determined by α-GST immunoblot (data not shown). *Indicates positions of unknown resin-associated polypeptides that are not detected by α-GST antibody.
(b) Autoradiograph of S^{35}-labeled ASK1 protein bound to GST, GST-LOV, GST-FL, GST-FKLCH and GST-FmutKLCH. ASK1 input lane represents 20% of the S^{35}-labeled protein used in the binding assay.

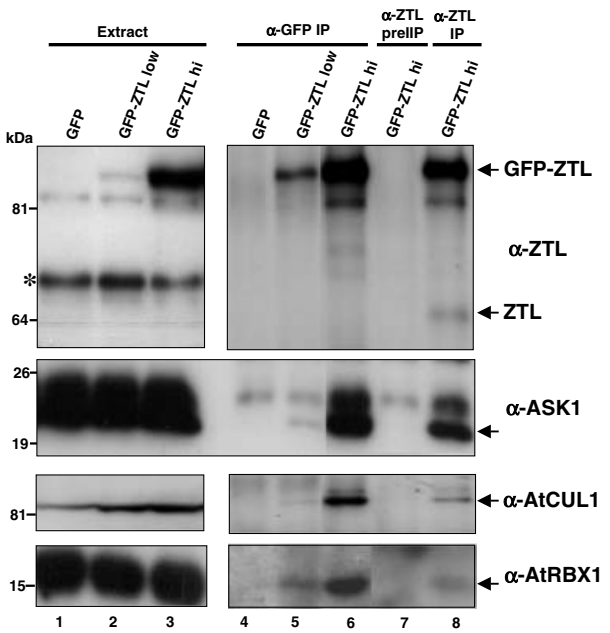

Figure 3. ZEITLUPE (ZTL) assembles into an Skp/Cullin/F-box (SCF) complex *in vivo*.
Total protein extracts from *Arabidopsis* seedlings expressing green fluorescent protein (GFP) alone (lane 1), GFP-ZTL at low (lane2) or high levels (lane 3) were co-immunoprecipitated with α-GFP antibody (lanes 4–6). Protein extracts from *Arabidopsis* seedlings with the highest GFP-ZTL protein level were co-immunoprecipitated with α-ZTL pre-immune serum (lane 7) and α-ZTL antibody (lane 8). Protein extracts and all resulting immunoprecipitates were immunoblotted and probed with the indicated antibodies. Arrows indicate the position of the appropriate band. *Indicates position of an unknown α-ZTL-detectable polypeptide (Kim *et al.*, 2003).

1998). We reasoned that ZTL may homodimerize, facilitated by the LOV/PAS domain. We probed the GFP-ZTL immunoprecipitates with ZTL antibody, expecting to detect native ZTL protein if GFP-ZTL/native ZTL dimers form *in vivo*. We did not detect any signal in the position where native ZTL migrates (Figure 3, lane 6). We tested immunoprecipitates from dark-adapted extracts (1 and 3 h after lights off; ZT 13 and ZT 15 respectively) with similar results (data not shown) and conclude that ZTL does not homodimerize *in vivo*.

F-box requirement for in vivo *association of ZTL with SCF components*

We expressed truncated versions of ZTL *in planta* to determine which domains are required for SCF complex formation. Plants stably expressing an N-terminally *myc*-tagged F-kelch protein (myc-Fkelch; residues 191–609) or an N-terminally *myc*-tagged kelch protein (myc-kelch; residues 244–609) under the control of the 35S promoter were identified (Figure 4a). The α-myc antibody was used to immunoprecipitate extracts obtained from both lines and F-kelch and kelch protein were appropriately identified using α-ZTL antibody (Figure 4a). We further tested the α-myc immunoprecipitates for the presence of ASK1 and cullin. Only in lines expressing the F-kelch protein were either of the two components detected in the immunoprecipitates (Figure 4a).

We then tested the ability of the N-terminus alone (LOV; residues 1–193) or the N-terminus plus the F-box (LOV-F; residues 1–284) to associate into an SCF complex. C-terminally myc-tagged proteins were stably expressed *in planta* under the control of the 35S promoter (Figure 4b). The α-myc antibody was neither able to immunoprecipitate nor able to detect on immunoblots either protein from plant extracts (data not shown). However, the α-ZTL antibody was able to both immunoprecipitate and detect on immunoblots both forms of truncated ZTL protein, and was used for subsequent analysis (Figure 4b). ASK1 was undetectable in immunoprecipitates from either of the two deletion lines (data not shown). However, cullin was clearly able to co-immunoprecipitate with overexpressed LOV-F protein, but not with LOV protein (Figure 4b).

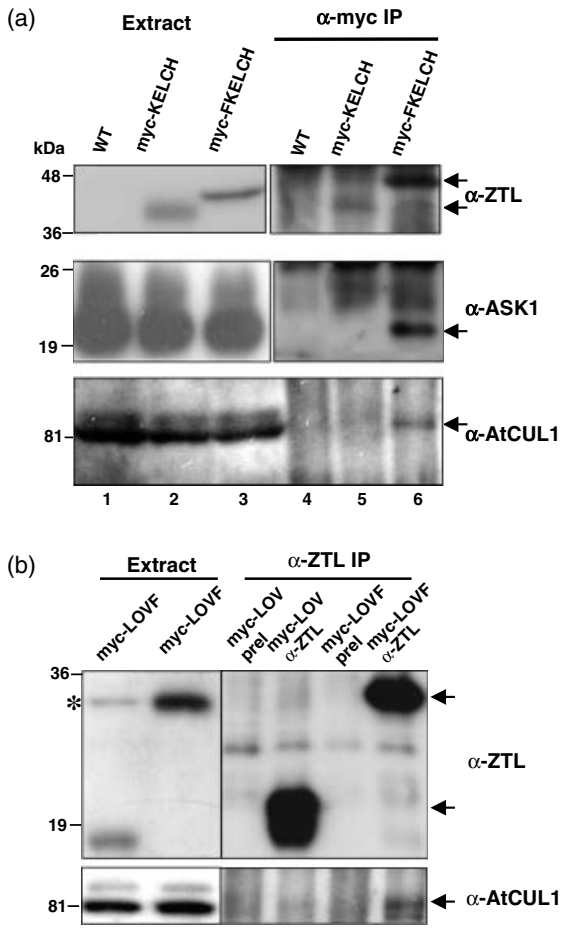

Figure 4. The C terminus of ZTL (FKELCH) is sufficient to form an Skp/Cullin/F-box (SCF) complex in *Arabidopsis*.
(a) Protein extracts from wild type (WT, lane 1) and plants expressing myc-tagged kelch and myc-tagged F/kelch polypeptides (lanes 2 and 3) were co-immunoprecipitated with α-myc antibody (lanes 4–6). Extracts and immunoprecipitates were detected with the indicated antibodies.
(b) The F-box is critical for the assembly of SCF complex *in vivo*. Protein extracts from WT and plants expressing myc-tagged LOV and myc-tagged LOVF polypeptides were co-immunoprecipitated with α-ZTL pre-immune and α-ZTL antibody. Extracts and immunoprecipitates were detected with the indicated antibodies. Arrows indicate the position of the appropriate bands. *Indicates position of an unknown α-ZTL-detectable polypeptide also present in WT (not shown) extracts.

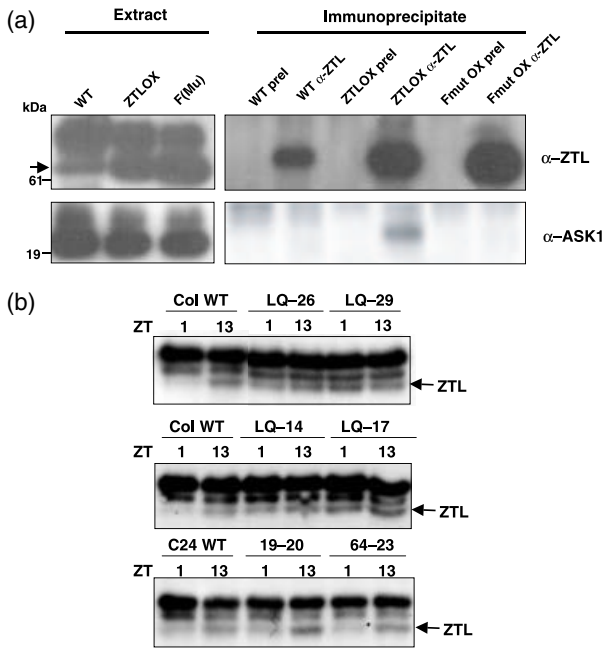

Figure 5. ZEITLUPE (ZTL) F-box mutations abolish *in planta* interaction between ASK1 and ZTL and stabilize the protein.
(a) Protein extracts from wild type (WT) and transgenic lines overexpressing full-length WT ZTL protein (ZTL OX) or full-length ZTL harboring F-box point mutations (Fmut OX) at comparable levels were immunoprecipitated with α-ZTL (α-ZTL) or pre-immune sera (prel) and detected with α-ZTL or α-ASK1 sera.
(b) ZTL accumulation at ZT 1 (1 h after lights-on) and ZT 13 (1 h after lights-off) from four independent Fmut low overexpression lines (LQ series) was compared with Col WT and two independent ZTL low enhanced expression lines (19–20 and 64–23 in C24 ecotype) from plants grown under 12-h light/12-h dark cycles. All arrows indicate ZTL position.

Altered circadian function by loss-of-binding F-box mutations

As a functional test of the requirement of the F-box for ZTL function *in planta*, we overexpressed a version of FL ZTL protein into which we introduced the two F-box amino acid replacements which had been effective in eliminating yeast two-hybrid interactions (see Figure 1). We identified transformants (Columbia ecotype) expressing this mutant protein (Fmut) at levels similar to that present in an overexpressor of FL wild-type ZTL (ZTL OX) (Figure 5a). Using α-ZTL antibody, the F-box mutation eliminated the ability to co-immunoprecipitate ASK1 with ZTL (Figure 5a).

Strong overexpression of ZTL causes arrhythmicity, while lower levels of enhanced ZTL expression shortens the circadian period (Somers *et al.*, 2004). Strong overexpression of the Fmut protein also results in arrhythmicity (data not shown). This might suggest that the F-box mutations have diminished but not completely eliminated the ability of Fmut protein to interact with ASK(s), and at very high levels sufficient amounts of SCFmut complexes form to affect the circadian period. Alternatively, we identified four transgenic lines that express the Fmut protein at levels similar to or slightly lower than two previously described lines (19–20 and 64–23) that express ZTL only 1.5–2-fold higher than wild type (WT) (Somers *et al.*, 2004)(Figure 5b). In contrast to the markedly shorter periods of two enhanced expressers of wild-type ZTL, the four Fmut lines had free-running periods 2–3 h longer than WT (Table 1). These results show that when Fmut protein is expressed at near-WT levels it disrupts normal circadian

Table 1 Effect of ZEITLUPE (ZTL) F-box mutations on free-running period

Genotype	Period (±SD)	*n*
Col WT	24.3 ± 0.2	13
Fmut-14	27.1 ± 0.3	19
Fmut-17	26.3 ± 0.5	22
Fmut-26	26.8 ± 0.5	30
Fmut-29	27.8 ± 0.4	24
C24 WT	24.3 ± 0.3	18
19–20	22.2 ± 0.2	26
64–23	22.5 ± 0.2	27

Four independently isolated lines (Col ecotype) constitutively expressing full-length ZTL containing two amino acid substitutions in the F-box (Fmut), and two lines expressing full length wild-type ZTL at comparable levels (C24 ecotype) were entrained for 6 days in 12 h white light/12 h dark cycles, then imaged under constant red light (30 μmol m^{-2} sec^{-1}) for 5 days. Variance-weighted period and SD estimates obtained as previously described.

function. The increased period length may result from a dominant negative interaction with upstream signaling components, causing a loss in the effectiveness of the endogenous ZTL still present in these backgrounds.

In addition, the three to fourfold higher level of ZTL at ZT13 (1 h after lights off), relative to ZT1 (1 h after lights on), typically seen in the WT (Kim *et al.*, 2003; Somers *et al.*, 2004), is absent in the four Fmut lines (Figure 5b). The F-box mutations have eliminated or reduced the normal degradative processing of ZTL at ZT1, resulting in a relative stabilization of the mutant protein and an accumulation to levels similar to that at ZT13.

Transient reduction in RBX1 phenocopies ztl *loss-of function mutations*

To further establish the importance of the SCF complex to proper circadian cycling we tested the effect of the transient loss of RBX1 on rhythmic expression of CCA1. RBX1 is a core component of the SCF complex of TIR1 and COI1 and appears to be the primary RBX component in plant SCF complexes (Gray *et al.*, 2002; Lechner *et al.*, 2002). Loss of RBX1 is lethal, so we employed the use of a dexamethasone (DEX)-inducible RNAi line to transiently reduce the level of RBX1 (Lechner *et al.*, 2002). Plants were entrained in light/dark cycles for 5 days, sprayed with 10 μM DEX and returned to one additional light/dark cycle before sampling every 3 h in constant light for 72 h. The level of RBX protein decreased detectably in response to DEX application and this drop was sustained over the sampling period (Figure 6a). Control plants (empty vector) sprayed with DEX showed robust rhythmic changes in CCA1 protein levels with a period near 24 h (Figure 6b). In contrast, the period of CCA1 cycling lengthened markedly in the DEX-inducible RNAi line (Figure 6b). This period lengthening is similar to that observed in *ztl* loss-of-function mutations (Somers *et al.*, 2000) and supports the notion that ZTL participates in the formation of an SCF complex (SCF^{ZTL}) that is essential to the proper regulation of the circadian period in plants.

Discussion

ZEITLUPE was isolated as a factor in the control of the circadian period. Based on sequence alignments, ZTL was predicted to act as an F-box protein and participate in SCF-dependent proteolysis (Somers *et al.*, 2000). When we identified ASK1 as a ZTL-interacting factor in a yeast two-hybrid screen we were able to further test biochemically and genetically the full involvement of ZTL in E3-dependent proteolysis. Our results confirm that ZTL is part of an SCF complex (SCF^{ZTL}) composed, at least, of AtCUL, ASK1 and RBX1, and provide evidence of direct association between an SCF complex and the control of the circadian period.

The F-box is required for SCF^{ZTL} *formation*

The association between ZTL and ASK1 is dependent on the presence of the F-box. Deletions of the F-box or mutations in conserved residues eliminate the interaction between ZTL and ASK1 in yeast two-hybrid tests, *in vitro* and *in planta*. F-kelch protein was able to immunoprecipitate from plant extracts with ASK1 and CUL1, demonstrating that the amino terminus of ZTL is unnecessary for SCF complex formation. Surprisingly, we were unable to co-immunoprecipitate ASK1 with the LOV-F polypeptide despite the very large amount of LOV-F protein stably present in the plant, and despite the ability to identify CUL1 in the immunoprecipitates. This suggests that another ASK may be forming a link between LOV-F and CUL1. This would be consistent with the implications of the reported co-crystallization of an Skp2/Skp1 complex (Schulman *et al.*, 2000). These authors conclude that the F-box protein (Skp2) is recruited to the complex by Skp1, in part, through the specific-substrate interaction domains of the F-box protein. For ZTL this domain is, in part, the kelch repeats (Mas *et al.*, 2003), and suggests that the F-kelch deletion protein might be recruited to one or more SCF complexes, and at least one class of complex would include ASK1. In contrast, the LOV-F protein may participate in other SCF complexes that do not include ASK1, but do contain the common CUL1 element.

Functional requirement of SCF^{ZTL} *for circadian cycling*

We took two functional approaches to establish that ZTL participates in the formation of an SCF E3 ubiquitin ligase complex that controls circadian cycling. One approach was to observe the effects on period of the loss or reduction in previously identified SCF components. We tested *ask1-1* and *ask2-1* homozygous single mutants and individuals of an F2

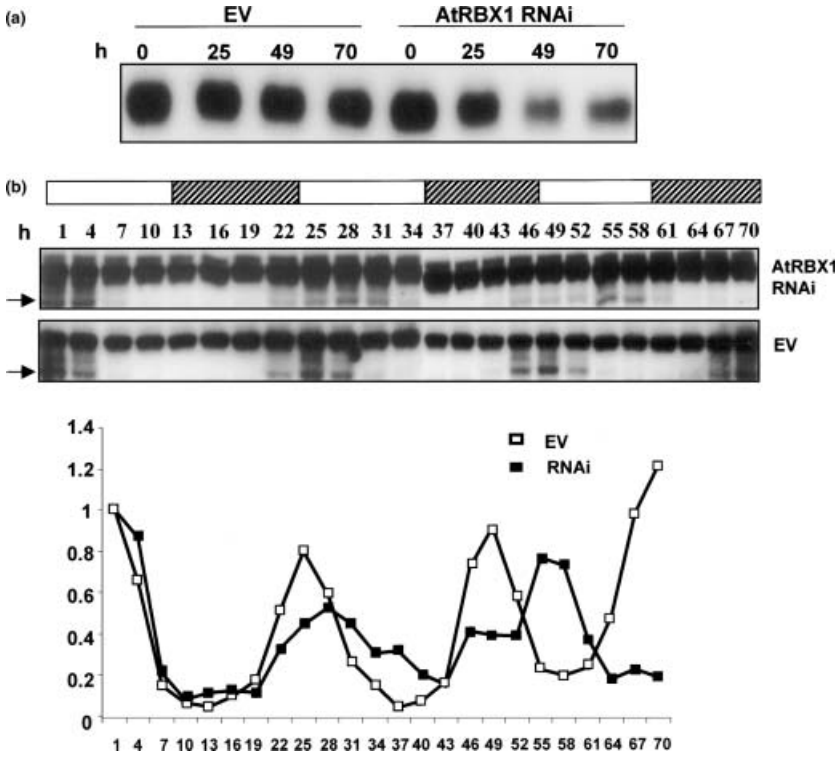

Figure 6. Reduced AtRBX1 levels alter circadian period.
(a) AtRBX1 protein level is reduced in the AtRBX1 RNAi line after dexamethasone (DEX) treatment. *Arabidopsis* seedlings from both empty vector (EV) and AtRBX1 RNAi lines were sampled before DEX treatment and at different time points during the free-running period as indicated in (b). Total proteins extracts (15 μg) were immunoblotted with α-RBX1 antibody.
(b) *Arabidopsis* plants expressing glucocorticoid-inducible AtRBX1 RNAi or transformed with EV were entrained under 12:12 LD cycles for 6 days, sprayed with 10 μM DEX, and entrained for an additional 12:12 LD cycle. Plants were transferred to constant light for 3 days and harvested at the indicated time. Protein extracts (30 μg) were immunoblotted and detected with α-CCA1 antibody. Quantitation is shown below of a representative of two trials. Arrows indicate CCA1 position. White and hatched boxes indicate periods of subjective light and subjective dark respectively.

population of the cross between them for circadian phenotypes. In no cases could we detect any effects on circadian period in constant red light (data not shown). As we are able to co-immunoprecipitate ZTL with ASK1 and ASK2, these results suggest some degree of redundancy in the recruitment of ASKs into the SCF^{ZTL} complex, as has been similarly suggested for SCF^{COI1} (Xu *et al.*, 2002).

As the primary element linking the F-box protein to the ubiquitin ligase activity of the SCF complex, ASK proteins may act as additional determinants to establish the target specificity of a given SCF complex. However, although *Arabidopsis* possesses 21 *ASK* genes (Farras *et al.*, 2001; Risseeuw *et al.*, 2003; Zhao *et al.*, 2003) only ASK1 has a discernible mutant phenotype, although *ask1 ask2* double mutants show phenotypes more severe than the *ask1* mutant alone (Liu *et al.*, 2004). ASK1 or a rice homolog has been previously identified as an *in planta* component of four other plant SCF E3 ligase complexes: SCF^{TIR}, SCF^{COI1}, SCF^{UFO} and SCF^{GID2} (Gomi *et al.*, 2004; Gray *et al.*, 1999; Wang *et al.*, 2003; Xu *et al.*, 2002). None of the F-box mutant phenotypes of auxin responsiveness (TIR1), jasmonate signaling (COI1) or flower development (UFO) is fully phenocopied by the *ask1* null mutant (Yang *et al.*, 1999). These data and ours support the notion that more than one ASK participates in mediating the association of ZTL with the complete SCF complex, with either redundant or partially redundant functions masking the effect of single or double *ask* mutants.

We next tested the effect of the transient loss of RBX1, a component that we have identified in SCF^{ZTL} (Figure 2) and has been shown to be part of the SCF^{TIR1} and SCF^{COI1} E3 ligases (Gray *et al.*, 2002; Xu *et al.*, 2002). We found that within 36 h after a reduction in RBX1 free-running period is lengthened. This result phenocopies a *ztl* null mutation (Jarillo *et al.*, 2001; Somers *et al.*, 2004), indicating that the loss of access to an SCF complex, either through the absence of the appropriate F-box component (i.e. ZTL) or from the loss of a common SCF element (i.e. RBX1), compromises proper regulation of the circadian cycle. Although the resolution is only at the 3 h level, the 27–30 h period estimated for the RBX1 mutant is very similar to the 27–28 h period of the *ztl* null mutant (Somers *et al.*, 2004). These data suggest that ZTL acts as the sole F-box protein, or possibly together with other F-box proteins in a single, common complex, to control the circadian period via an SCF-type E3 ubiquitin ligase. The *ztl-1 fkf1* double mutant shows a *ztl-1* period phenotype (data not shown), confirming that the closely related F-box protein, FKF1, does not participate in the control of the circadian period (Imaizumi *et al.*, 2003).

The second approach correlated the loss of the weak ZTL overexpression phenotype (shortened period) with loss of the ability of ZTL to form an SCF^{ZTL} complex because of mutations in the F-box (Fmut). Surprisingly, period lengthened in the presence of near-WT levels of Fmut, causing a phenocopy of loss-of-function *ztl* mutations. The Fmut protein may act as a dominant negative polypeptide, unable to form into a functional SCF complex, and competitively inhibiting other complex formations required for proper ZTL function. Overproduction of

N-terminal portions of the Cdc4p and Met30p F-box proteins inhibit SCF complex function in yeast (Dixon *et al.*, 2003), and the presence of a form of ZTL that is unable to interact with ASK may act similarly. *In vitro* and yeast two-hybrid data suggest that phytochromes and/or cryptochromes may interact with ZTL *in planta* (Jarillo *et al.*, 2001). Interactions between these photoreceptors and Fmut could sequester them into non-productive complexes and phenocopy photoreceptor mutants, which are also long period (Devlin and Kay, 2000; Somers *et al.*, 1998). An alternative explanation is that essential ZTL homodimerization is disrupted by the Fmut protein. However, this is less likely, as our evidence indicates that ZTL does not homodimerize *in planta*.

The F-box is required for ZTL degradation

We have previously shown that ZTL undergoes proteasome-dependent degradation at specific circadian phases (Kim *et al.*, 2003). The stabilization of a ZTL mutant protein lacking a functional F-box (Figure 5b) now suggests that this degradation of ZTL depends on its interaction with the components of an active SCF complex. These data can now be coupled with our findings that a point mutation in the kelch domain of *ztl-1* eliminates binding of the TOC1 substrate (Mas *et al.*, 2003), but the mutant protein levels still oscillate like WT in light/dark cycles (Somers *et al.*, 2004). Similarly, strong constitutive overexpression of TOC1 has no effect on steady-state ZTL levels, or on its rhythmic changes in light/dark cycles (W. Y. Kim and D. E. Somers, unpublished data). Together, these results suggest that ZTL regulation is mostly dependent on SCF complex participation alone, independent of substrate availability. These data differ from previous reports implicating both SCF participation and substrate presence as key regulators of the stability of the F-box protein, HOS (Galan and Peter, 1999; Li *et al.*, 2004).

SCF^{ZTL} substrates

F-box proteins are the substrate specifiers of the SCF complex. In principle, there could be a unique one-to-one relationship between an F-box protein and a substrate; however, a single F-box protein can target more than one substrate for degradation (Deshaies, 1999; Gray *et al.*, 2001). Recent evidence implicates TOC1 as a ZTL substrate (Mas *et al.*, 2003). Under entraining light/dark cycles, TOC1 levels rise to a maximum during the middle of the dark, start to fall during the latter part of the night, and reach a minimum during the early light period. However, in extended light (LL) TOC1 cycling damps to high levels, and conversely, TOC1 damps low in extended dark (DD). In *ztl* mutant backgrounds TOC1 levels stabilize to peak levels at all time points in both light and dark, suggesting that ZTL-mediated TOC1 degradation is not only dark-dependent, but also time-delayed under light/dark cycles. TOC1 belongs to a five-member gene family of *PRR*, all of which can affect the circadian period when disrupted or overexpressed (Eriksson *et al.*, 2003; Ito *et al.*, 2003; Kaczorowski and Quail, 2003; Michael *et al.*, 2003; Sato *et al.*, 2002; Yamamoto *et al.*, 2003). Additionally, the CONSTANS gene family is involved in photoperiodic timing and shares an extensively similar protein domain with the PRR family (Strayer *et al.*, 2000). Hence, considerations from the perspective of both protein function and similarity recommend the likelihood of other ZTL substrates.

Experimental procedures

Plasmids and constructs

Yeast two-hybrid

PCR-based amplicons were made using the ZTL cDNA (Somers *et al.*, 2000) and the primers

(1) 5′-CCCGGGGGAGTGGGACAGTGGT-3′
(2) 5′-GTCGACGGCTCTCTTTGGTTA-3′
(3) 5′-CCCGGGTTCCCGAGGAATGTG-3′
(4) 5′-CTGCAGAGCTGAGTATATCCC-3′
(5) 5′-GGATCCGAAGTGAAACAACACGGGTA-3

to generate FL (residues 1–609; primers 1 and 2), FKLCH (residues 191–609; primers 3 and 2) and kelch (residues 248–609; primers 5 and 2) products that were ligated into pBTM116 (Clontech, Palo Alto, CA, USA) as translational fusions to the LexA binding protein.

The double point mutations in the F-box (Fmut; L200 to A, L213 to A) were generated by PCR mutagenesis (Michael, 1994) using two mutagenic primers (L200A: 5′-TGGGTTATTCCAGGCGAGTGACGAGGTTGT-3′; L213A: 5′-AACGTCTCTTGGTGTCGCGCGTGACAATA-3′) nested within two flanking primers (primer 3 and Fmut-R: 5′-GGGAGCTCACTTTGACATG-3′). Products were scored by restriction digest (*Hha*I and *Pvu*II) to identify the double mutant and cloned into pBTM116.

GST fusions. ZTL cDNA sequence was subcloned from pBTM116/FL as an *Sma*I/*Xho*I fragment into pGEX-KG (Guan and Dixon, 1991) to make the GST-FL ZTL construct, and similar compatible digests and reclonings from the pBTM116/FKLCH and pBTM116/FmutKLCH to pGEX were performed to make the GST- FKLCH and GST-FmutKLCH constructs respectively. For the GST-LOV construct (residues 1–192), an *Sma*I/*Bso*BI fragment was prepared from pBTM116/FL, followed by Klenow fill-in and ligation into pGEX-KG.

GFP fusions. The GFP-ZTL FL gene fusion was constructed in pRTL2 (Restrepo *et al.*, 1990) by recombinant PCR (Higuchi, 1990) using primer 6 (5′-GGTCGCCACCATGGTGAGCAAGG-3′) and primer 7 (5′-CCACTGTCCCACTCCATCTTGTACAGCTCGTC-3′) with pEGFP-1 (Clontech) as template, and primer 8 (5′-ACGAGCTGTACAAGATGGAGTGGGACAGTGG-3′) and primer 9 (5′-CTCTCCCGGGTTACGTGAGATAGCTCGC-3) with the ZTL cDNA as template. The final FL gene fusion was directionally cloned into pRTL2 at *Nco*I/*Sma*I restriction sites and confirmed by sequencing. The 35S promoter-driven construct was subcloned into pZP221 (Hajdukiewicz *et al.*, 1994).

myc-*tagged fusions.* Full-length ZTL cDNA in pRTL2 (Somers *et al.*, 2004) was digested with *Xba*I and *Xmn*I to remove the

C-terminal region, and a single c-myc cassette (EQKLISEEDL) was cloned in to make a C-terminal tagged LOV-F-box-c-myc fusion construct (ZTL residues 1–284). Similarly, a *Bso*BI/*Xba*I digest removed all but the first 192 residues of ZTL to which c-myc was appended to create a LOV-c-myc fusion. An *Nco*I/*Bso*BI digest removed the first 190 ZTL residues and, separately, an *Nco*I/*Nsi*I digest removed the first 243 ZTL residues, into which single c-myc cassettes were cloned to create a c-myc-FKELCH fusion construct (residues 191–609) and a c-myc-kelch construct (residues 244–609) respectively. In all cases pairs of complementary c-myc oligomers (5′-GAGCAAAAGCTTATCAGTGAGGAGGATCTA-3′) with the appropriate restriction site sequence prefixed or appended were used to obtain the above fusions with ZTL.

GST pull-down assay

GST, GST-LOV, GST-FKLCH, GST-FmutKLCH and GST-ZTL were expressed in *E. coli* BL21(DE3) and purified with glutathione–agarose (Sigma, St. Louis, MO, USA). ^{35}S-labeled ASK1 protein was synthesized by using the T′n′T *in vitro* transcription-translation system (Promega) according to the manufacturer's instructions. *In vitro* binding assays were according to Sugano *et al.* (1998). In brief, equal aliquots (5 μl) of ^{35}S-labeled ASK1 were added to 50 μl of binding buffer [20 mM Hepes pH7.6, 100 mM KCl, 10% glycerol, 5 mM EDTA, 0.02% NP-40, 1 mM DTT, 5 mg ml^{-1} BSA and protease inhibitor cocktail (Sigma)]. Concentration of each fusion protein was determined by Coomassie staining. The amount of glutathione–agarose beads with bound GST, GST-LOV, GST-FKLCH, GST-FmutKLCH and GST-ZTL was adjusted so that equal amounts of fusion protein were present for the *in vitro* binding reactions. The final volume of *in vitro* binding reaction was brought up to 100 μl with distilled water and conducted at 4°C for 3 h. The beads were then washed four times with binding buffer and once with binding buffer without BSA. The bound protein was eluted in 1x SDS sample buffer and size-fractionated on 12% SDS-PAGE along with 0.5 μl ^{35}S-labeled ASK1 as input. Detection was by autoradiography.

Immunoprecipitation and immunoblotting

Immunoprecipitations were performed according to Kim *et al.* (2003). All extracts were from plants grown under 12-h light/12-h dark cycles and harvested 7 h after lights on (ZT 7) unless otherwise noted. The appropriate antibodies [α-GFP rabbit polyclonal antibody (Molecular Probes, Eugene, OR, USA), 1:1000; affinity-purified α-ZTL antibody [Kim *et al.*, 2003], 1:250; α-myc antibody (9E10) (Santa Cruz Biotechnology, Santacruz, CA, USA), 1 μg] were incubated with 50 μl protein A agarose (Invitrogen, Carlsbad, CA, USA) made to a final volume of 100 μl with co-IP buffer (Kim *et al.*, 2003) at 4°C for 4 h overnight. One to 2 mg of protein extracts were added to the affinity matrix and incubated at 4°C for 1 h with gentle rotation. Immune complexes were harvested at 785 ***g*** for 3 min at 4°C. Complexes were washed three times with 1 ml ice-cold co-IP buffer and once with PBS buffer, resuspended in 50 μl 2x SDS-PAGE sample buffer, boiled and resolved (10 μl) by SDS-PAGE. Immunoblot analysis was performed at room temperature for 2 h according to Kim *et al.* (2003), using membranes incubated with the appropriate primary antibody [affinity-purified α-ZTL antibody, 1:1000; α-ASK1 antibody, 1:10 000; α-RBX1 (human) (Biosource, Camarillo, CA, USA), 1:2000; α-AtCUL, 1:5000]. Following secondary antibody incubation (1 h) and washing, detection was by ECL (Amersham, Piscataway, NJ, USA). Immunoblot detection of ZTL and CCA1 was according to Somers *et al.* (2004) except 50 μg (ZTL) and 35 μg (CCA1) protein was loaded per lane.

Plant growth and rhythm analysis

Plant growth conditions and methods of rhythm analysis were as described by Somers *et al.* (2004).

Transgenic plant materials

The glucocorticoid-inducible AtRBX1 RNA interference (RNAi), AtRBX1 overexpressor and empty vector control lines were as described by (Lechner *et al.*, 2002). Plant transformations were conducted as described in Somers *et al.* (2004).

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