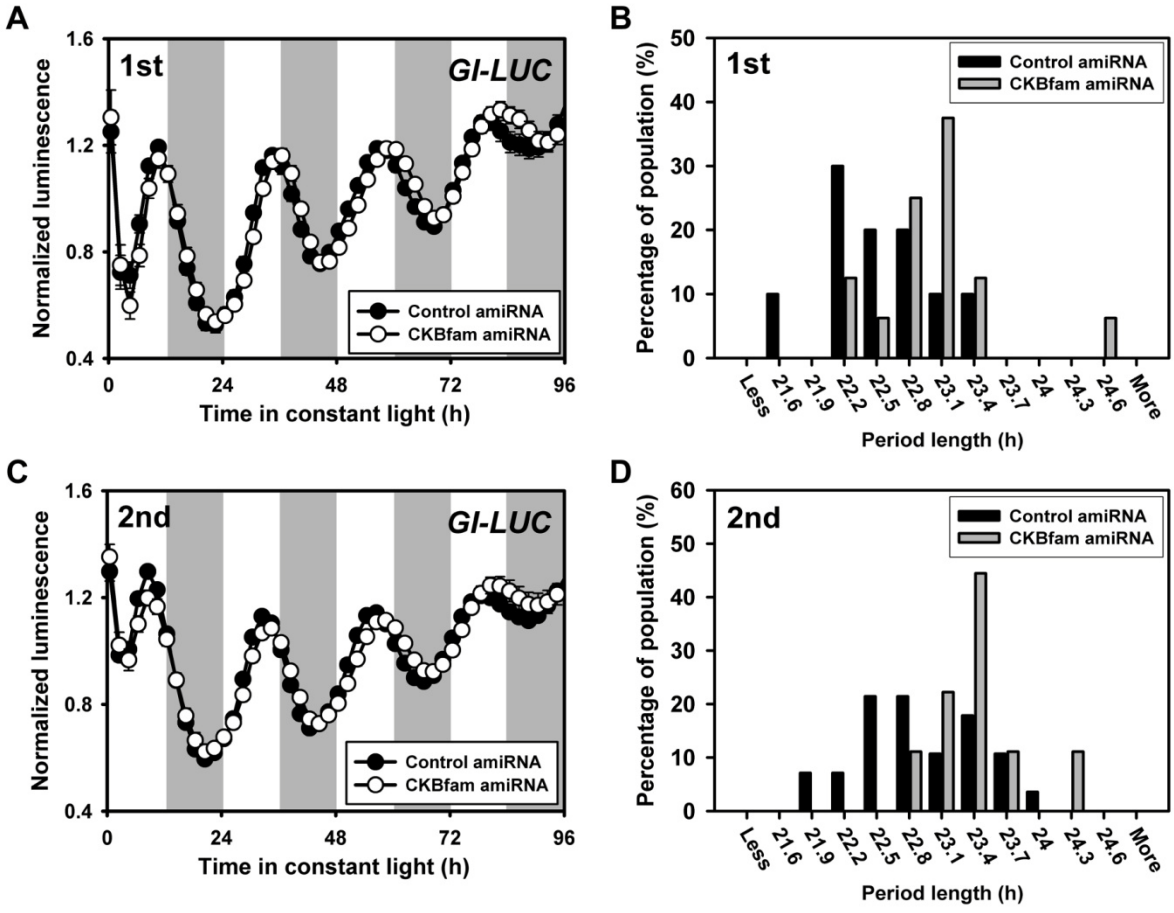

Figure S1. amiR-CKBfam delays circadian period in transgenic plants. A and C, The average traces of bioluminescent readouts of *GI-LUC* in T1 transgenic lines harboring control and CKB amiRNA. B and D, The population distribution of the period lengths of T1 transgenic lines harboring control amiRNA and amiR-CKBfam. Experiments and period estimations were performed as described in Figure 5A, except T1 transgenic lines were used and grown on the media containing hygromycin. Each data set was normalized to the mean expression level over the 0 to 96 h sampling schedule. Data represent mean values ±SEM ([1st trial] n=10 for control amiRNA and n=16 for amiR-CKBfam [2nd trial] n=28 for control amiRNA and n=9 for amiR-CKBfam. White and gray regions indicate subjective light and dark period. Two biological trials were shown in (A,B) and (C,D), respectively.

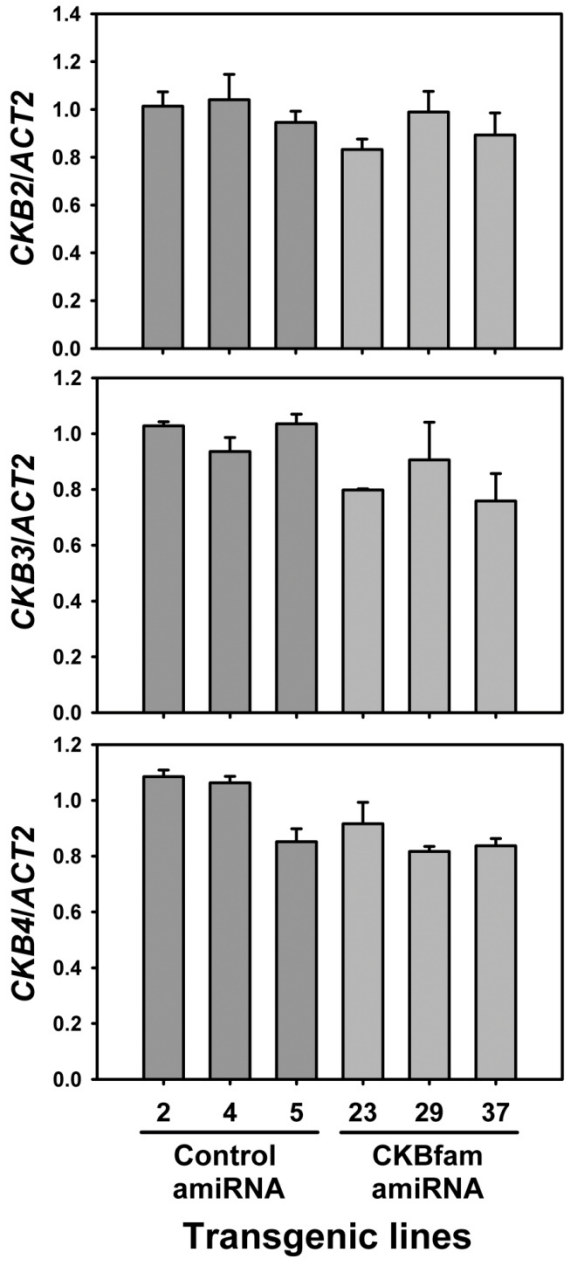

Figure S2. Relative expression of *CKB2*, *3* and *4* in transgenic lines harboring CKB amiRNA. Transgenic lines were entrained under 12-h-light/12-h-dark cycles for 10 d and harvested at ZT 9. Gene expression was determined as described in Figure 3B. Data represent mean value ±SEM (n=2).

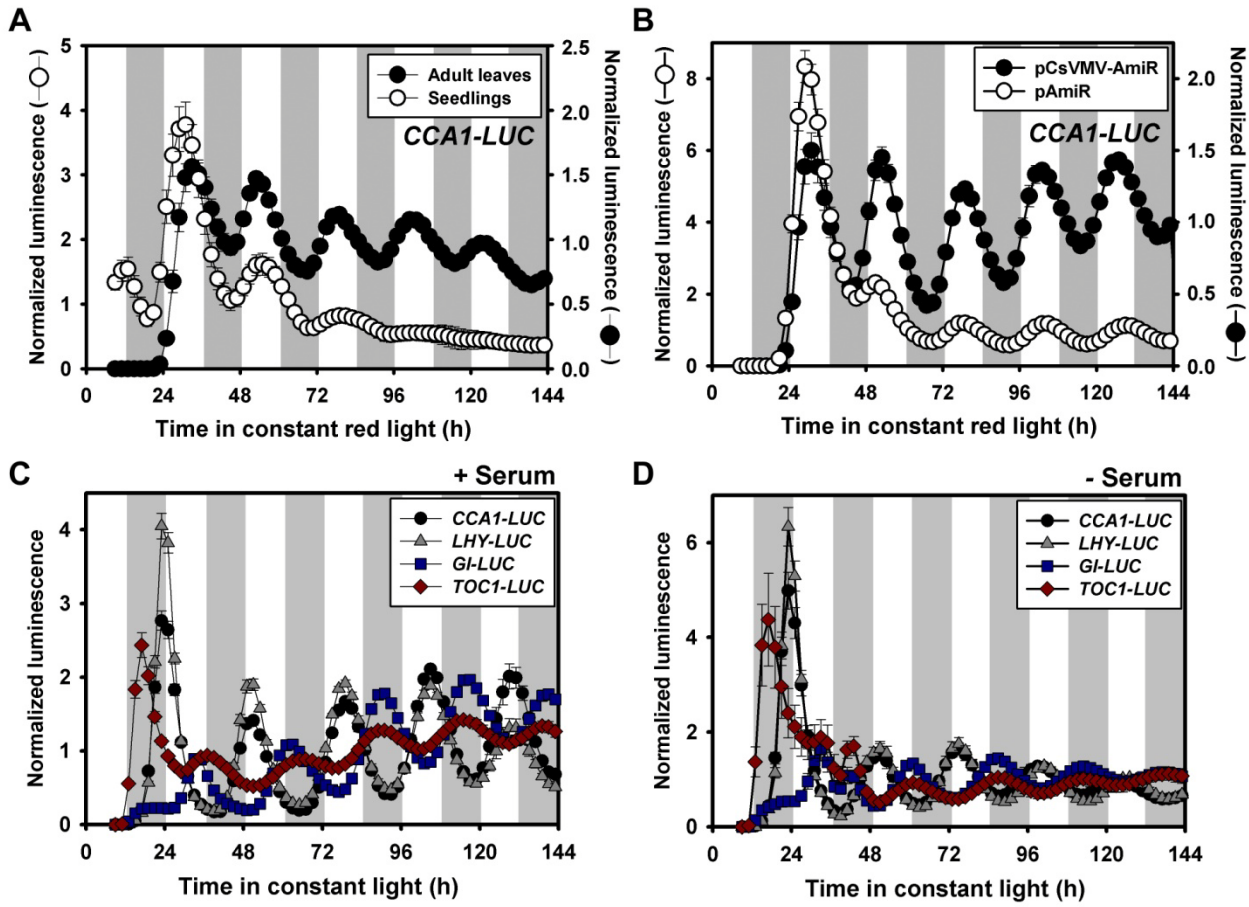

Figure S3. Optimization of clock-controlled luminescence cycling in Arabidopsis protoplasts.

A, The average traces of bioluminescent readouts from *CCA1-LUC* transfected protoplasts extracted from adult rosette leaves and seedlings. Protoplast preparations and transfections using adult leaves were performed as described in Figure 1, but using *CCA1-LUC* and pCsVMV-amiRNA plasmids. Seedling protoplasts were prepared from 10 to 14-d-old seedlings grown on sterile MS media containing 3% sucrose under a 12L/12D cycle and transfected with *CCA1-LUC* and pCsVMV-amiRNA control plasmid. Transfected protoplasts were resuspended in W5 incubation buffer with 5% fetal bovine serum and transferred to constant red light at ZT4. Data represent mean values ±SEM (n=4). Similar results were obtained in two independent trials. B, The average traces of bioluminescent readouts from protoplasts transfected using either pCsVMV-AmiR or pAmiR control vectors in conjunction with the *CCA-LUC* reporter plasmid. Experiments were performed as described in Figure 1. Data represent mean values ±SEM (n=6). Similar results were obtained in two independent trials. C and D, The average traces of bioluminescent readouts from protoplasts transfected with clock controlled promoter (*CCA1*, *LHY*, *GI* and *TOC1*) driven luciferase, incubated in W5 buffer containing 5% fetal bovine serum (+ Serum; C) and W5 buffer only (-Serum; D). Experiments were performed as described in Figure 1, but using different incubation buffers as indicated. Data represent mean values ±SEM (n=6). Similar results were obtained in two independent trials.

Table S1. Calculated interaction likelihoods between amiRNAs and potential target gene(s).

amiRNA	amiRNA core sequence (5'→3')	amiRNA source[a]	Potential target gene(s)	Target recognition sequence (3'→5')[b]	Hybridization energy (kcal/mol)[c]	Perfect-match-dG cutoff (70%)[d]
amiR-ELF3	GGCTTGTATATCAGTCCTTGCGGGGGA	A	*ELF3*	GGCTTGTATATCAGTCCTTCCGAGGGA	-57.1	87%
amiR-GI	TATTGCCAAAATTCGGCGCCT	W2	*GI*	GATTGCCAAAATTCGGCGCGT	-38.3	87%
amiR-ZTL	GACTTATATAATAGGTTGGTCGGGCTC	A	*ZTL*	GACATATATAATAGGTTGGTCCGGCTC	-48.4	83%
			LKP2	AACATAAATGATAGGGTTGTCAGGTTC	-23.2	40%
			FKF1	GACATAGATCAAAGGAAAATCTGGCTC	-25.5	44%
amiR-CKBfam	TTAACGAGCATGAATCAGACC	W2	*CKB1*	GTAACGAGCATGAATCAGTCC	-36.3	84%
			CKB2	GAAACGAGCATGAATCATTCC	-32.1	75%
			CKB3	GTAACGAACATGAATAAGACC	-32.9	76%
			CKB4	ATAACGAACATGAATAAGACC	-33.1	77%
amiR-CKB1	TAAGTCTGATTGACGAACCGG	W3	*CKB1*	TAAGTCTGATTGACCAACCGG	-39.6	89%
			CKB2	GATATCGGATTGACCAACTGG	-22.9	52%
			CKB3	GATATCGGATTGTCCAACTGG	-18.1	41%
			CKB4	GATATCAGATTGGCCAACCGG	-24.1	54%
amiR-CKB2	TTCTTTACGGTGCTCGCTCTA	W3	*CKB2*	ATCTTTACGGTGCTTGCTCTA	-38.0	84%
			CKB1	ATCTTCACAGTGCTAGATCGC	-20.0	44%
			CKB3	ATCTTCACAGTACTCGATCTC	-17.6	39%
			CKB4	ATCTTCACAGTGCTCGACCTC	-24.0	53%
amiR-CKB3	TTTTAAGTTCCCGTAAGTCAA	W3	*CKB3*	CTTTAAGTTCCCGTAAGTCAT	-37.8	100%
			CKB1	CTTGAGATGCCCATAAGTCAT	-22.5	60%
			CKB2	CTTCAGATGCCCGTACGTCAT	-22.3	60%
			CKB4	TTTCATGTTCCCATAGGCCAT	-25.9	69%
amiR-CKB4	TGGAACGTAGTTTTGCGCCGT	W3	*CKB4*	AGGAACGTAGTTTTGAGCCGG	-37.2	81%
			CKB1	TTGAACATAGTTTTGTGTTGC	-23.3	51%
			CKB2	TTGAGTGTAGCTTTGTGATGC	-17.3	38%
			CKB3	TGGGACATAGCTTTGAGTAGG	-20.3	44%

[a] A, From ABRC; W2 and W3, Designed using the WMD2 and 3 (web microRNA designer2 and 3), respectively.
[b] Matched sequences between amiRNA and target sequence are underlined.
[c] Hybridization of amiRNA to its mRNA target site was calculated using the RNAcofold program at the WMD2 website.
[d] Ratio of the hybridization energy between amiRNA and its target to that between amiRNA and its perfect reverse complement in percent.

Supplemental Results and Discussion

Optimization of assay conditions for circadian rhythm measurements in Arabidopsis mesophyll protoplasts

Standard procedures for protoplast isolation and transfection with plasmids necessarily include stress-inducing procedures like cell-wall digestion and phase-resetting environmental treatments like dark and cold treatment (for details see Methods). In addition, circadian assays require a prolonged time course to accurately estimate circadian clock parameters. Originally we adopted the standard procedures of (Yoo et al., 2007) in our experiments which require protoplast extraction for two and half hours under darkness and protoplast stabilization on ice for 30 min. These treatments have little or no effect on the robustness of cyclic luminescence expression even though we observed a slight phase shift (data not shown). However, prolonged measurement of LUC activity for 6 days also requires ethanol treatment of detached leaves and further handling of protoplasts under sterile conditions.

To optimize the procedure for sustained and robust rhythms we tested other possible factors, such as plant materials, vector systems, and incubation conditions. First, we tested two source materials, seedlings and adult leaves, which have been used as sources for protoplast isolation in the past. Although using Arabidopsis seedlings have the advantage of not requiring ethanol treatment to surface sterilize the material, the overall rhythmic expression patterns of *CCA1-LUC* in leaf mesophyll protoplasts were more sustainable and with higher amplitudes and sharper peaks than results derived from seedling protoplasts (Supplemental Fig. 3A).

Next, we compared two vectors for expressing amiRNA in protoplasts, pAmiR and pCsVMV-AmiR. pAmiR is the backbone used for the generation of the genome-wide amiRNA plasmids deposited at the ABRC, and is suitable for expression studies in protoplasts. We tested pAmiR-based amiRNA plasmids from the ABRC collection for *ELF3*, *GI*, and *ZTL* genes and found effects on circadian rhythms and on the corresponding target gene expression similar to those shown in Figures 2 and 3 (data not shown). However, in these studies the luminescence rhythms (*CCA1-LUC*) showed an unusually sharp peak in the first day of incubation, and relatively weak amplitudes with wide peaks during further prolonged incubation, even when only the pAmiR backbone was used (Supplemental Fig. S3B). These effects were remarkably absent when using the *CCA1-LUC* reporter plasmid alone (Fig. 1, A and C) or when co-transfected with

the pCsVMV-AmiR (Supplemental Fig. S3B). In addition, the cassava vein mosaic virus promoter drives stronger expression in Arabidopsis mesophyll protoplasts than does the 35S promoter (Kim and Nam, unpublished). Therefore, we replaced the vector backbone from pAmiR with pCsVMV-999 (cassava vein mosaic virus promoter; (Kim et al., 2008; Verdaguer et al., 1998) by transferring the amiR foldback region into pCsVMV-999 to create pCsVMV-AmiR. Compared with the pAmiR plasmid, the pCsVMV-AmiR vector generated relatively consistent, robust and reliable rhythms of *CCA1-LUC* expression for more than 6 days (Supplemental Fig. S3B).

Finally, we examined how the luminescence rhythm patterns depended on incubation conditions, which is important for the prolonged incubations necessary for circadian clock experiments. We tested the LUC-based rhythm in various conditions, including different incubation buffers (W5; see Methods, and WI: 500 mM Mannitol, 20 mM KCl, and 4 mM MES-KOH [pH 5.7]), plastic plates with/without calf serum coating and different light intensities. One of the most important incubation conditions we observed is the addition of 5% fetal bovine serum (F4135, Sigma) to W5 incubation buffer (Yang, 1985). Without inclusion of the serum luminescence levels are lower, amplitude is lower and oscillations tend to rapidly dampen out, especially when amiRNA plasmids are co-transfected (data not shown). We consistently found that adding serum to the incubation buffer improved the luminescence rhythm assay in protoplasts (compare Supplemental Fig. S3, C and D).

Thus, to obtain reliable and reproducible data for these assays, we measured luminescence rhythms by co-transfecting protoplasts extracted from Arabidopsis leaves with the pCsVMV-AmiR vector and *CCA1-LUC* plasmid, followed by incubation in W5 buffer containing 5% fetal bovine serum. These conditions were used for all experiments in the main figures.

Supplemental Materials and Methods

Plasmid construction

To create *LHY-LUC* and *TOC1-LUC* reporters, the 5' upstream region encompassing the *LHY* and the *TOC1* promoter was amplified by PCR with Pfu DNA polymerase, using Arabidopsis

Columbia genomic DNA as a template and the following sets of primers : *LHY* (5'-TTT GGA TCC GGT CTA CAG TGT GAT TGG ATC ACT -3' and 5'-TTT AGG CCT AAC AGG ACC GGT GCA GCT ATT C -3') and *TOC1* (5'-TTT GGA TCC CCA TAG AAG GGA CTC TCT CCT GAC -3' and 5'-TTT AGG CCT GAT CAG ATT AAC AAC TAA ACC CAC -3'), respectively. The underlined nucleotides indicate restriction enzyme sites. The resulting PCR products were digested with *Bam*HI and *Stu*I, and ligated into pCR-CCD-F entry vector (Kim and Nam, unpublished). The final *LHY- LUC* and *TOC1-LUC* constructs were established by LR recombination reaction (GATEWAY) using each corresponding entry clone and the Gateway version of pOmegaLUC vector (Kim and Nam, unpublished).

Seedling protoplast transfection with amiRNA and reporter plasmids

Seedling protoplast isolation were performed according to (Yoo et al., 2007) with minor modifications. All procedures were carried out in the clean hood. Seedling protoplasts were prepared at ZT12 (i.e. twelve hours after lights-on) from 10 to 14-d-old seedlings grown in sterile MS media containing 3% sucrose under a 12L/12D cycle. The seedlings were soaked in 7 mL of an enzyme solution (400 mM Mannitol, 20 mM KCl, 20 mM MES-KOH [pH 5.7], 10 mM $CaCl_2$, 1% Cellulase R10, 0.5% Macerozyme R10, and 0.1% BSA) in a 60-mm diameter Petri dish, and minced with a sterile razor blade. This enzyme digestion was performed in the dark, with gentle shaking (35 rpm) for 12 hours at room temperature. Subsequent handling was identical to the leaf protoplast protocol found in Experimental Procedures.

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