Supplemental file 1

A daily expression pattern of protein-coding genes and small non-coding RNAs in *Synechocystis* sp. PCC 6803

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Supplemental Results

The total protein amount of the culture kept in light/dark (LD), of the culture transferred to constant light (LL) and of the culture transferred to constant darkness (DD) remained nearly constant during the 48-h time series experiments; (in pg/cell) LD: 2.65 ± 0.12, LL: 2.27 ± 0.07, DD: 2.74 ± 0.12.

The ratios of phycocyanin to chlorophyll *a* (PC/Chl*a*) remained constant during the 48-h time series experiments; LD: 0.801 ± 0.004, LL: 0.827 ± 0.003, DD: 0.811 ± 0.002.

Supplemental Methods

RNA preparation, cDNA synthesis and Quantitative Real-Time PCR. We used quantitative real-time PCR to validate our microarray analysis. The TRIzol extraction kit (Invitrogen) was used for RNA extraction as detailed in Material and Methods of the main text. The RNA was then subjected to treatment with TURBO DNase (Ambion) and confirmed by PCR to be free of detectable amounts of DNA. Five micrograms of RNA were reverse-transcribed with the Maxima First Strand cDNA Synthesis Kit for qRT-PCR (Fermentas) according to the manufacturer's instructions. Quantitative RT-PCR was performed in 96-well plates (Applied Biosystems) using *Power* Sybr(R) Green PCR Master Mix (Applied Biosystems) and a 7500 Real-Time PCR System (Applied Biosystems). Gene-specific primer pairs were used to measure the accumulation of *kaiA1*, *kaiC1*, *kaiC2*, *kaiB3*, *kaiC3*, *cikA*, *rbcL*, *ftsZ*, and *atpH* transcripts; primer sequences are listed below (see Fig. S2 for qRT-PCR results). For each primer pair, a standard curve based on serial dilutions of the cDNA was included. Each 10 µl-reaction contained 1X buffer, 1.8 pmol of each primer and amounts of cDNA as template, corresponding to 0.001-1.0 ng of reverse transcribed total RNA. Due to the very low transcript levels of the *kaiA1*, *kaiC1*, *kaiC2*, *kaiB3*, and *kaiC3* genes, amplification was performed in a 20 µl-reaction volume using 10-100 ng of reverse transcribed total RNA. The specificity of each primer pair was tested by a melting curve analysis. The accumulation of each transcript was analyzed in duplicate. Values displayed in Fig. S2 represent means of duplicates. The transcript analysis was done on LD and LL time

course cultures used for Microarray analysis. Non-template control reactions were performed for each primer pair. Negative controls (omitting reverse transcriptase) were included on all samples to verify the complete removal of DNA. The raw data were analyzed using the Sequence Detection Software v1.4 (Applied Biosystems) and crossing points determined by second derivative maximum analysis. The accumulation of transcripts were normalized to the amount of *rnpB* RNA (LD) and *trnM* RNA (LL) as internal standards by the ΔCT method (2-ΔCT [ΔCT = CT target – CT internal standard]). The calculations were performed within the linear range of the amplification curves of reference and target genes (Sequence Detection Software v1.4, Applied Biosystems).

A list of primers used for quantitative real-time PCR analysis can be found below. Primer sequences are given in 5'→3' orientation.

Transcript	Primer 1	Primer 2
kaiA1 (slr0756)	TTTCTCAAATCCTGGAAATTCAC	GCTCCGCCCTTCTAGCTT
kaiC1 (slr0758)	TTTACCAATACCACGGACCAA	GCAACATCAAAATGGTGTCTGT
kaiC2 (sll1595)	GCCCTAGAACTCAGCAAACG	TTGACTGGCTAGTTTCATTTGG
kaiB3 (sll0486)	GGGTAATTCGGTGCGTTCT	TCAACATTGTACTGATCCCCTAGA
kaiC3 (slr1942)	TCAAGGGATGCACATTGGTA	AAACCGCTCAATCTTTCCAC
cikA (slr1969)	ACTGTCCCAGCTTGAATCCG	CCCCCAACAGGGAGTGAATC
ftsZ (sll1633)	GTGGAACCGATCTGACCCTG	TCGTCAATCACCGCTCCAAA
rbcL (slr0009)	CCGCTGGCACCTGCGAAGAA	GGCAGTGAAACCGCCGGTGA
atpH (ssl2615)	GATCGGCCCTGGTATTGGTC	AAAAGCCAGGGTCAACAGCA
trnM-CAU	GGCTTGGTAGCTCAGTTGG	TCGGGGCGGATTTGAACC
rnpB	GGAAGCAAGGTCGGAGGGGC	ATGCGAGGCACCCTTGGGGA

Protein quantification. Every 4 hours, 20 ml of each cell culture were sampled for protein extraction and quantification every four hours. The cells were harvested by centrifugation at 3,900 x g for 5 min at ambient temperature. Pelleted cells were frozen in liquid nitrogen and stored at -20°C. 1 ml cell suspension was frozen in liquid nitrogen and stored at -20°C. After thawing, TCA was added to a final concentration of 10% and samples were incubated on ice for 20 min followed by 10 min centrifugation (15,000 x g, 4°C). Precipitated proteins were dissolved in 0.5 ml 1M NaOH, incubated at room temperature for at least overnight and

centrifuged for 1 min at 2,200 x g. 0.1 ml of the supernatant was used for protein quantification according to Lowry (1).

Whole-cell extract preparation, SDS-PAGE and Western Blot analysis. For western blot analysis, an independent time series experiment was performed. The experimental conditions were identical to those described in the main text. A synchronized *Synechocystis* culture was kept under a 12:12-hours light/dark cycle for 24 hours. Every 2 hours, cells from 15 ml culture were harvested by centrifugation at 3,900 x g for 5 min at ambient temperature. Pelleted cells were frozen in liquid nitrogen and stored at -20°C. After re-suspension in ice cold thylakoid buffer (50 mM Hepes/NaOH, pH 7.5 mM $MgCl_2$, 25 mM $CaCl_2$, 50 mM EDTA, pH 8, 10% (v/v) Glycerol) supplemented with a phosphatase inhibitor cocktail (Roche) and protease inhibitor cocktail (Roche), cells were disrupted with an equal volume of glass beads (0.1 mm and 0.25-0.5 mm, 1:1 mixture) in a bead mill (Reetsch) at 4°C. Protein concentration in the resulting whole-cell extracts were determined using the Lowry method (1) including precipitation with sodium deoxycholate and trichloroacetic acid according to Bensadoun and Weinstein (2). For each time point whole-cell extracts, containing equal amounts of total protein were separated by Tricine–SDS-PAGE (3) and blotted onto a PVDF membrane by semi-dry blot transfer. The membranes were blocked with 5% milk in TBST (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween) and incubated overnight at 4°C with antibodies to detect 30S ribosomal protein S1 (Synpcc7942_0694) (Agrisera, 1:5000 in TBST) or 50S ribosomal protein L1 (Synpcc7942_0633) (Agrisera, 1:10000 in TBST), followed by incubation for 1 hour at room temperature with corresponding peroxidase-conjugated goat anti-rabbit secondary antibody, IgG (Sigma, 1:50000). Bands were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Millipore) and analyzed by Molecular Imager ChemiDoc XRS+ (Bio-Rad). Using the ImageLab software (Bio-Rad), the chemiluminescence signals from triplicate Western blot analyses were quantified relative to the signal obtained at 5.5 hours. In parallel, proteins were separated by Tricine–SDS-PAGE and stained with Coomassie-blue to control the protein amount in the samples used for western blot analysis. Signals from Coomassie-stained gels were quantified relative to the signal obtained at 5.5 hours. The values from the Western blots were set into the ratio with the relative values of the total protein amount. The quantitative analysis was performed in the linear range of protein detection by the antibodies. The resulting data were plotted relative to the average level of the whole measurements (Fig. S3).

Supplemental Figures

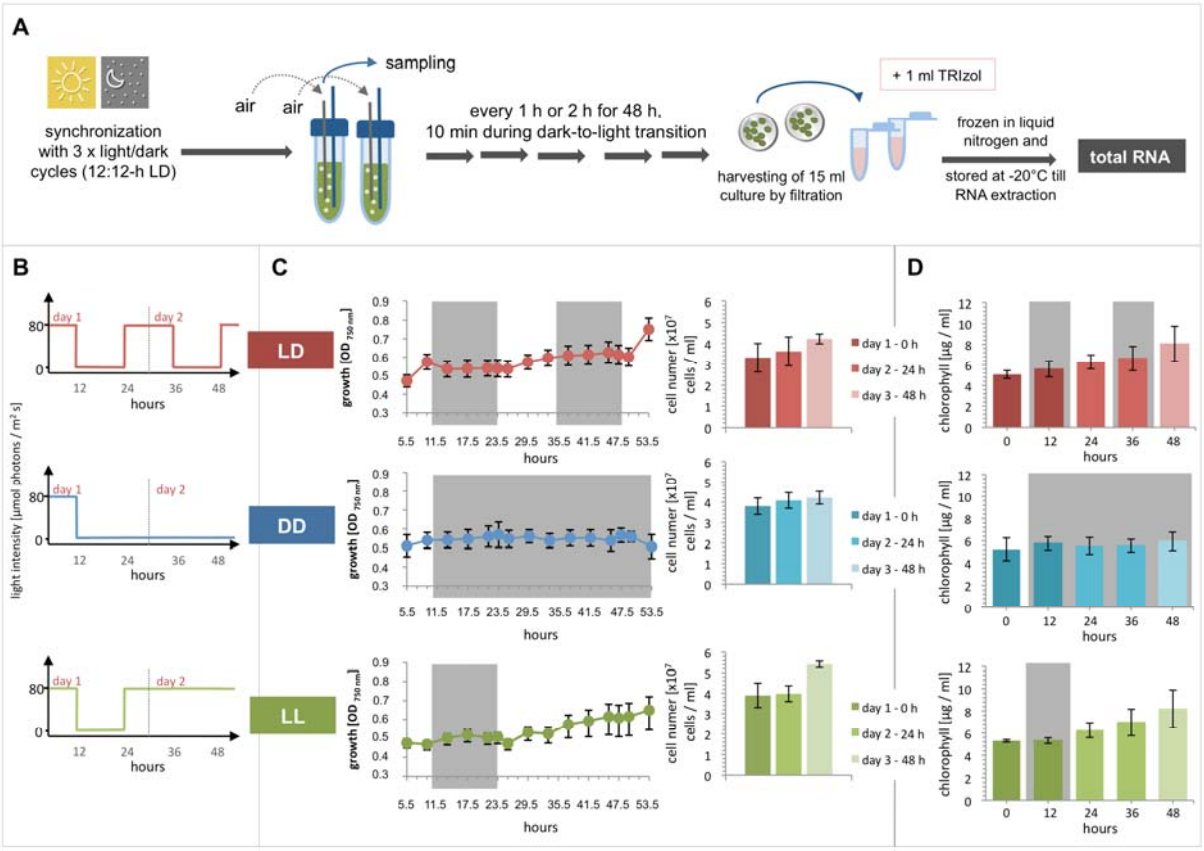

Figure S1 Experimental set-up and collecting of physiological data. (A) Sampling strategy. *Synechocystis* sp. PCC 6803 batch cultures were grown in moderate light intensity (80 µmol of photon/m^2s) at 30°C and stirred by a constant stream of air. After 3 days of synchronization to a 12:12-h light/dark cycle, the cultures were diluted up to a certain volume and an OD_{750nm} of approximately 0.4. (B) Light conditions. Cultures were kept under three different light conditions during the 48-h sampling. One culture was kept in LD (red), while a second culture was transferred to DD at 12 hours (blue) and a third culture to LL at 24 hours (green). Samples for total RNA extraction were taken at 2-h intervals and additionally, around the dark-to-light transition, at 10 to 30 minute intervals. (C) Control of cell growth. The growth rates over the 48-h period were determined by measuring the optical density at 750 nm every 2 hours (line plots) as well as by manual cell counting at 0 hours, 24 hours and 48 hours (bar plots). The growth of cells slightly increases in the culture kept in LD and in the culture transferred to LL. The growth of cells rests in the DD culture. (D) Control of pigmentation. In accordance with the cell growth, the chlorophyll amount of the cultures increases slightly in LD and LL whereas it remains constant in DD. In summary, the physiological data suggest that all three cultures were kept under unstressed conditions. For each of the three time series, the data are means ± standard errors from the results of four independent experiments. Dark phases are indicated in gray.

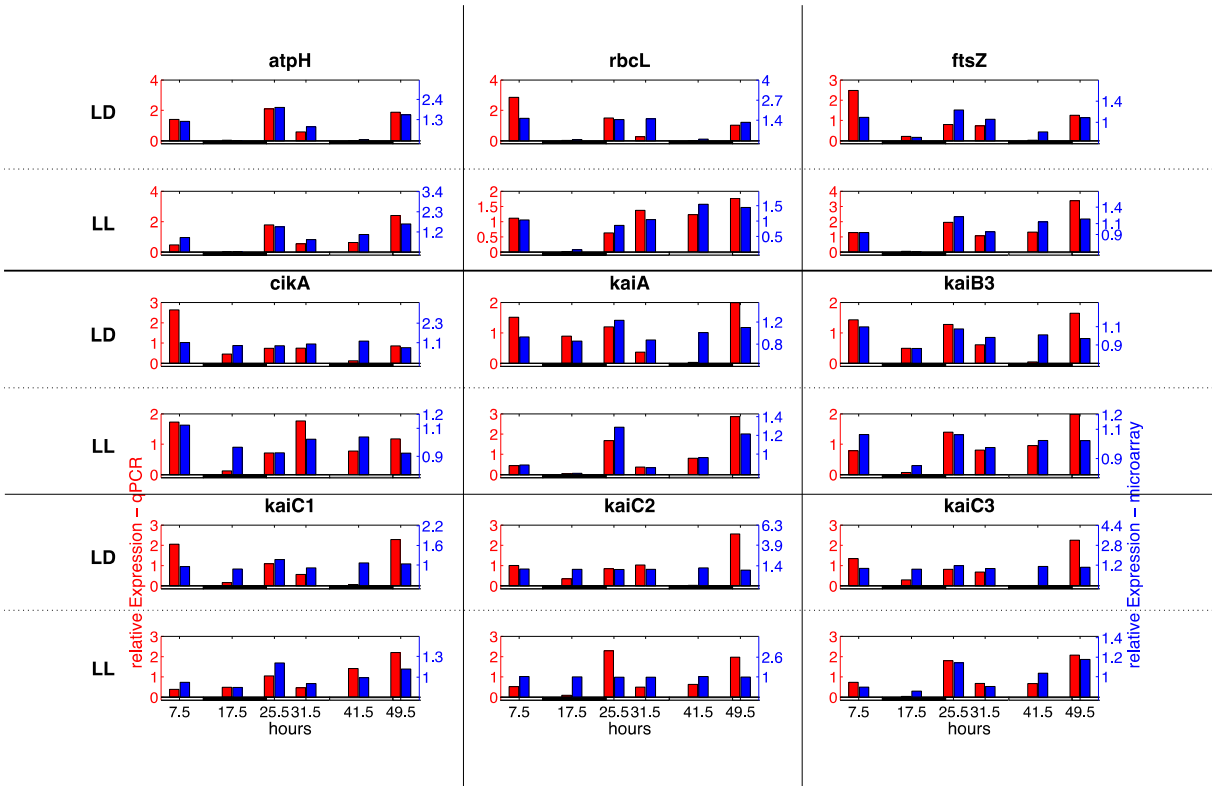

Figure S2 Quantitative real-time PCR validation of microarray data. Example mRNA accumulation profiles of nine genes under light/dark rhythm (LD) and after shifting to constant light (LL) were determined by microarray and quantitative real-time PCR analysis. A good agreement was obtained between qRT-PCR and microarray data for transcripts with medium accumulation (top row): *atpH* mRNA (encoding an ATP synthase subunit), *rbcL* mRNA (encoding the large subunit of RubisCo), and *ftsZ* mRNA (encoding a cell division protein). Differences between the two methods were observed for transcripts with very low accumulation (below the black line), such as the transcripts of the clock genes *kaiA*, *kaiB3, kaiC1, kaiC2,* and *kaiC3* as well as the clock-associated *cikA* gene (encoding a circadian input kinase). Here, the transcript amounts obtained by microarray analysis disappear into the background noise of the chips and should be treated with caution. By contrast, the qRT-PCR analysis can detect even very small transcript amounts. In summary, the transcript amounts of all nine genes analyzed cycle under light/dark periods but lose any rhythm after the shift to LL regardless of light-regulated genes (*atpH*, *rbcL*), clock genes (*kai* genes) or clock-associated genes (*ftsZ*, *cikA*). Furthermore, the accumulation of each transcript in the subjective night phase (indicated by a gray bar in the LL series) resembles always that of the light phase and not the expression level during the second night phase of the LD series. White and black bars indicate light and dark period; gray bars indicate virtual night during the continuous illumination. The mRNAs of the *rnpB* gene (LD) and of the *trnM-CAU* gene (LL) were used for data normalization in the quantitative real-time PCR analysis.

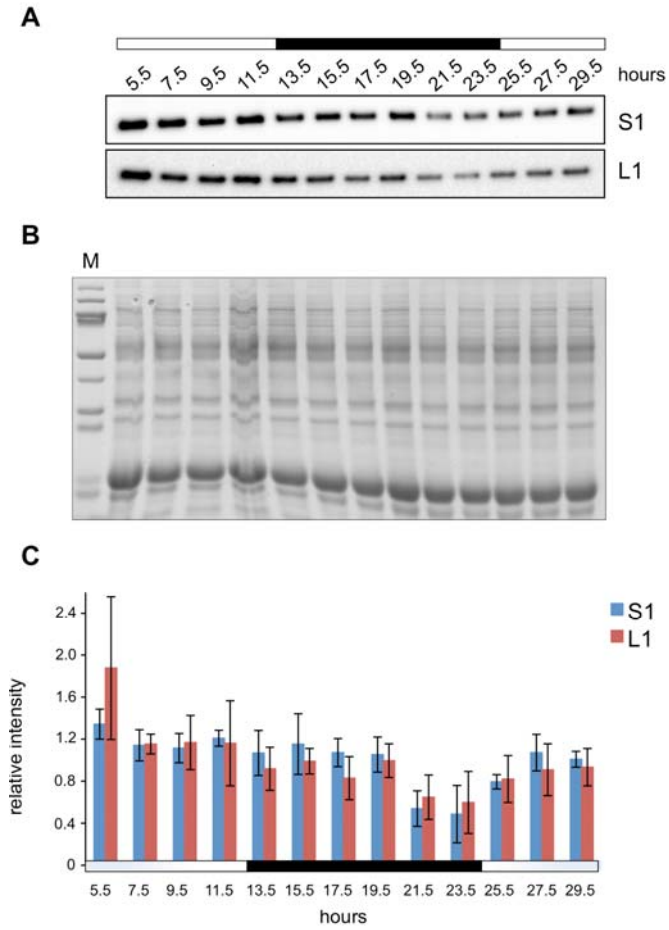

Figure S3 Analysis of ribosomal protein abundance in *Synechocystis* sp. PCC 6803 under 12:12-h light/dark rhythm. (A) Western blots with 50S ribosomal protein L1 and 30S ribosomal protein S1 antibodies. Total protein was extracted from cells. Protein concentrations were determined and equal amounts of protein (10 µg) were subjected to Western blot analysis. (B) Loading control. In each lane, 20 µg of total protein were separated by SDS-PAGE followed by Coomassie-blue staining. M denotes the marker lane. (C) Chemiluminescence signals from two Western blots (three technical replicates each for L1 and S1) were quantified and normalized by the average abundance level of L1 and S1. The data are means ± standard deviations. White and black bars indicate light and dark phases.

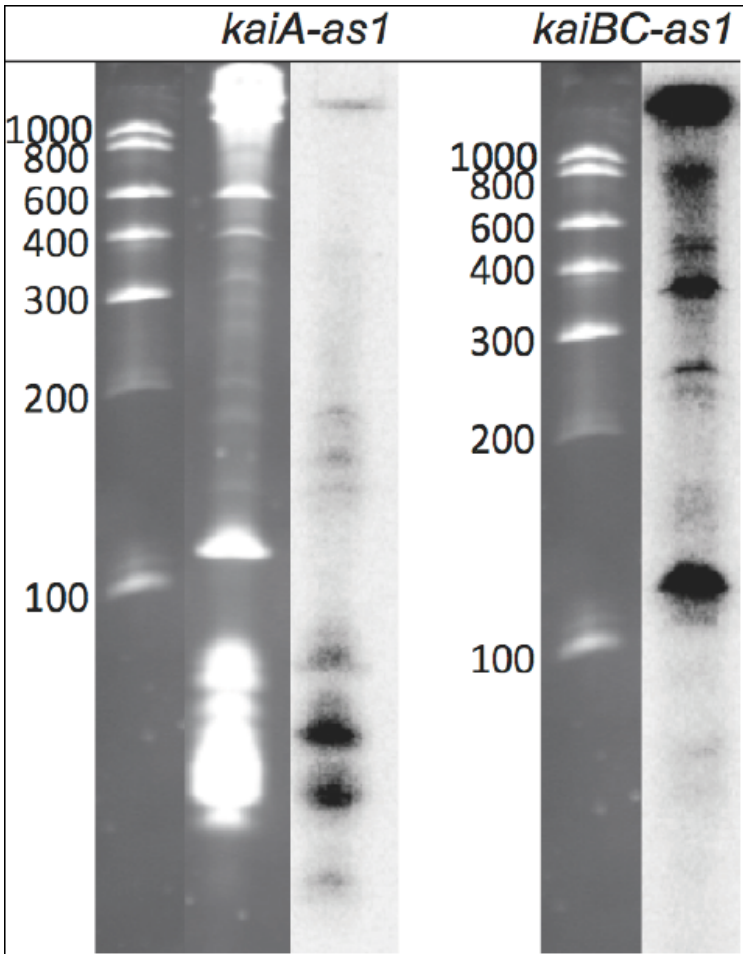

Figure S4 Northern Blot analysis of *kaiA* antisense and *kaiB1/kaiC1* antisense transcripts. Two Northern Blots were performed to verify the expression of *kaiA-as1* and *kaiBC-as1* transcripts found by microarray analysis.

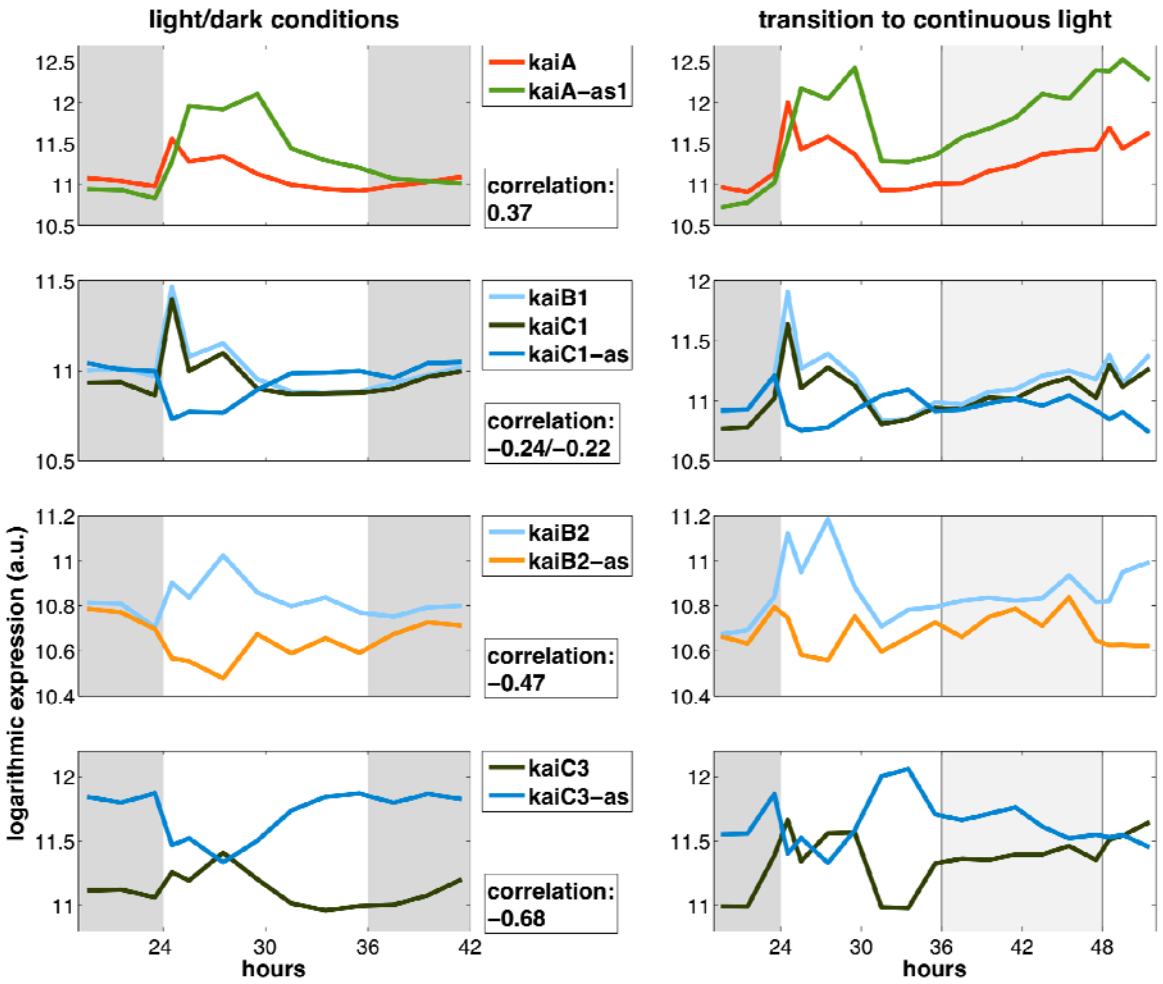

Figure S5 Analysis of the correlation between the accumulation dynamics of the *kai* mRNAs and that of their *cis*-encoded antisense RNAs in *Synechocystis* sp. PCC 6806. Shown are the accumulation profiles of selected *kai* mRNA/*kai* antisense RNA pairs in light/dark (left panels) and after shift of the culture to continuous light (right panels). **Left panels:** The accumulation dynamics of the antisense kaiA-as1 shows a positive correlation with that of its target transcript, indicating the possibility that this antisense RNA regulates positively the abundance level of the *kaiA* mRNA (first panel). The *kaiA*, *kaiB1* and *kaiC1* genes are arranged in tandem on the genome. Since the amounts of the *kaiA*, *kaiB1* and *kaiC1* mRNAs increase and decrease to the same degree, the antisense RNA kai-as1 might regulate accumulation of all three mRNAs (first and second panel). The antisense RNA kaiC1-as shows an opposite accumulation pattern compared to that of the *kaiC1* and *kaiB1* mRNA. Like kai-as1 but in a negative manner, kaiC1-as regulates the accumulation of the mRNAs of the *kaiA*, *kaiB1*, and *kaiC1* genes. The accumulation dynamics of *kaiB2* mRNA and kaiB2-as RNA are strongly anti-correlated to each other (third panel). The same is true for the accumulation dynamics of *kaiB3* mRNA and kaiB3-as RNA (fourth panel). The values of the correlation coefficients of both mRNA/antisense RNA pairs are high. The accumulation of mRNA and antisense RNA is the average of two replicates. For each accumulation dynamics

of mRNA and respective antisense transcript, the Spearman's rank correlation coefficient was computed. **Right panels:** The correlated accumulation patterns between mRNA and antisense RNA seen in light/dark are lost in constant light conditions. Gray areas indicate the 'real' night phases, while the light gray areas indicate the subjective night in continuous light.

Supplemental References

1. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ.** 1951. Protein measurement with the Folin phenol reagent. J Biol Chem **193:**265-275.
2. **Bensadoun A, Weinstein D.** 1976. Assay of proteins in the presence of interfering materials. Anal Biochem **70:**241-250.
3. **Schagger H, von Jagow G.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **166:**368-379.