### **Support Information**

## **SI Materials and Methods**

# **Plant Materials and Growth Conditions**

The *cop1-4, cop1-6* (1), *pif4-2* (2), *cca1-22* (3), *csu4* (*csu4-1* to *csu4-3*) (this study) mutants, *CCA1-OX* (4) and *PIF4-OX* (5) are in the Columbia-0 (Col-0) ecotype. Seeds were surface sterilized with 30% commercial Clorox bleach and 0.02% Triton X-100 for five min and washed three times with sterile water, and sown on  $1\times$ Murashige and Skoog (MS) medium supplemented with 0.8% Agar (Difco) and 1% sucrose. The seeds were stratified in darkness for three days at  $4 \degree C$ , and then transferred to light chambers maintained at 22 °C.

## **Genetics Screen, Identification, and Characterization**

Genetic complementation tests showed that two different *csu4* (*csu4-1 cop1-6* and *csu4-2 cop1-6* lines) ethyl methanesulfonate (EMS) mutations were allelic to each other. Homozygous mutant suppressor plants were crossed to wild-type plants (Col), and segregation in the  $F_2$  generations was analyzed in the dark to distinguish between intragenic and extragenic suppressors between two *csu4 cop1-6* lines. The ratio of wild-type or suppressed phenotype to  $\text{cop1-6}$  phenotype in the  $F_2$  population was around 13:3, indicating that two *csu4 cop1-6* lines are extragenic suppressors. The two extragenic suppressor mutants were backcrossed to  $\text{cop1-6}$ . F<sub>1</sub> generations showing the *cop1-6* phenotype and the segregation patterns in the  $F_2$  generations (suppressor phenotype: *cop1-6* phenotype≈1:3) in the dark confirmed that the suppression phenotype is caused by a monogenic recessive mutation in two *csu4* alleles.

## **Map-Based Cloning of** *csu4-1*

Rough mapping of *CSU4* was performed as described preciously (6, 7). Briefly, *csu4-1 cop1-6* (Col background) was crossed with *cop1-6*, which was backcrossed to Landsberg, eight times to produce the mapping population. F2 progeny seeds were grown on  $1\times$  Murashige and Skoog (MS) medium supplemented with 0.8% Agar (Difco) and 1% sucrose in the dark for 5 days. The suppressed-phenotype seedlings (long hypocotyl) were transferred to white light for additional 5 days and then picked for mapping. The markers used for mapping were designed based on the Arabidopsis Mapping Platform (http://amp.genomics.org.cn) and the standards described previously (8).

### **SOLiD Sequencing and Mutation Identification**

The procedures of SOLiD sequencing and mutation identification was described previously (6, 7) The fragment libraries were created using the SOLiD Fragment library construction procedures according to the manufacturer's instructions (Life Technologies). The libraries were sequenced on a SOLiD5500 sequencer according to the manufacturer's instructions (Life Technologies). Mapping of sequencing reads to the *Arabidopsis* reference genome (TAIR10) and single nucleotide polymorphism (SNP) calling were accomplished using LifeScope version 2.5. SNPs were then sorted into four categories (EMS-induced homozygous, EMS-induced heterozygous, other homozygous, other heterozygous). Candidate homozygous EMS-induced SNPs were identified in windows with reduced heterozygosity in the regions identified by physical mapping using in-house scripts.

### **Construction of Plasmids**

The full-length *CSU4* and *CCA1* coding sequences (CDS) were cloned into the pDONR-221 or pDORN-223 vector (Invitrogen), and introduced into the plant binary vect*or pEarlyGateway 104*, *pEarleyGateway 202*, or *pEarlyGateway 203* (9) under the 35S promoter using Gateway LR Clonase enzyme mix (Invitrogen). *pEarleyGateway-35S:YFP-CSU4*, *pEarleyGateway-35S:myc-CSU4* and *pEarleyGateway-35S:Flag-CCA1* were generated. To produce constructs for genomic complementation test, the promoter and genomic region of *CSU4* gene was cloned into *EcoRI*/*NcoI* sites of *pCambia1300* vector. To produce constructs for BiFC assays, the full-length *CSU4* or *CCA1* CDS were introduced in to *pSPYNE* and *pSPYCE* (10) using Gateway LR Clonase enzyme mix (Invitrogen). To produce constructs for yeast-one hybrid and yeast-two hybrid assays, 400 bp *ELF4* promoters upstream of *ATG* were amplified by PCR with the respective pairs of primers and then cloned into the *KpnI*/*XhoI* sites of the *pLacZi2µ* vector (11). *CSU4*, *CSU4 N* and *CSU4 C* CDSs were cloned into the *EcoRI/BamHI* sites of *pLexA* vector (Clontech). *CSU4* or *CCA1* CDS were cloned into the *EcoRI/HindIII* sites of *pB42AD* vector (Clontech). To produce constructs for firefly luciferase transient transfection assays, 1580 bp *ELF4* promoter or 946 bp *PRR9* promoter upstream of *ATG* were cloned into the *HindIII/NcoI* sites of *pGreenII 0800-LUC* vector (12). *CSU4* or *CCA1* CDSs were cloned in to the *EcoRI/XhoI* sites of pGreenII 62-SK vector (12). For the prokaryotic expression of CSU4, the *CSU4* CDS was cloned into the *BamHI/NotI* sites of *pET28a* vector (Novagene). *pCombia1300-35S:P19* (for suppressing PTGS) (13), constructs were described previously. *pLexA-CSU4 N, pB42AD-CCA1, pLacZi2µ-ELF4pro, pGreenII 62-SK-CCA1* and *pET28a-CSU4* constructs were constructed using digestion-ligation cloning method. *plexA-CSU4, pLexA-CSU4 C, pB42AD-CSU4, pGreenII 62-SK-CSU4, pGreenII 0800-LUC-ELF4pro* and *pGreenII 0800-LUC-PRR9pro* constructs were constructed using homologous recombination method with ClonExpress II One Step Cloning Kit (Vazyme). The primers used for plasmids construction were listed in Supplemental Table 1.

## **Genomic Complementation Test**

The *pCombia1300-proCSU4:CSU4* or *pEarley Gateway-35S:YFP-CSU4* constructs were transformed into *Agrobacterium tumefaciens* GV3101 by the freeze-thaw method, and then introduced into *csu4-1 cop1-6* or *csu4-2 cop1-6* plants via the floral dip method (14). Transgenic plants were selected on MS medium containing 20 mg/L hygromycin or 20 mg/L Basta respectively. T3 homozygous lines are used for phenotypic analysis.

## **Measurement of Hypocotyl Length**

To measure the hypocotyl length of seedlings, seeds were sown on plates and stratified at 4 °C in darkness for three days, and then kept in continuous white light for 8 h in order to induce uniform germination. The seeds were then transferred to dark, white, blue, red, and far-red light conditions, and incubated at 22°C for 4 days (15). The hypocotyl length of seedlings was measured using ImageJ software.

#### **Production of Specific CSU4 Polyclonal Antibody**

To generate a CSU4-specific antibody, the full-length of *CSU4* was cloned into pET28a. Recombinant 6xHis-CSU4 protein was expressed in the bacterial strain BL21 (DE3). The soluble recombinant 6×His-CSU4 protein was purified using nickel-nitrilotriacetic acid beads (Qiagen). Polyclonal CSU4 antibody was raised by immunizing rabbits using purified 6×His-CSU4 recombinant protein as antigen. The antibody was affinity-purified using the purified 6×His-CSU4 antigen protein immobilized on polyvinylidene difluoride membranes.

### **Co-immunoprecipitation (Co-IP) Assays and Immunoblot Analysis**

For Co-IP assays using *Nicotiana benthamiana* leaves, the *Agrobacterium* strain GV3101 carrying *35S:myc-CSU4* or *35S:FLAG-CCA1* constructs were transiently infiltrated into *Nicotiana benthamiana* leaves. The tobacco leaves were grown in the long-day conditions for 3 d and then lysed. The extracts were incubated with 4 µL of anti-myc antibodies (Sigma-Aldrich) coupled with 25 µL of Protein-A Sepharose (GE Healthcare) for 3 h at  $4^{\circ}$ C. The Sepharose was then washed three times with protein extraction buffer. The precipitates were eluted into 100 mM glycine (pH 2.5) and 100 mM NaCl, immediately neutralized by 2 M Tris·HCl pH 9.0 and 100 mM NaCl, and finally concentrated using StrataClean Resin (Stratagene) before immunoblot analyses.

For immunoblots, *Arabidopsis* WT or mutant seedlings were homogenized in a protein extraction buffer containing 100 mM NaH2PO4, 10 mM Tris·HCl, 200 mM NaCl, 8 M Urea, pH 8.0, 1 mM phenylmethylsulfonyl fluoride and 1×complete protease inhibitor cocktail (Roche). Primary antibodies used in this study were anti-CSU4 (this study), anti-cMyc (Sigma-Aldrich), anti-Flag (Sigma-Aldrich), anti-GFP (Abmart) and anti-Actin (Sigma-Aldrich).

#### **Bimolecular Fluorescence Complementation (BiFC) Assays**

The YFP<sup>N</sup> - and YFP<sup>C</sup> - fused plasmids were transformed into *Agrobacterium* strain GV3101, and the indicated transformants pairs were infiltrated into *Nicotiana benthamiana* leaves. A Carl Zeiss confocal laser scanning microscope (LSM510 Meta) was used to detect the YFP fluorescence signals. YFP fluorescence was excited by a 514 nm laser and detected between 517 nm and 589 nm.

#### **Yeast-one and-two Hybrid Assays**

For the yeast one-hybrid assay, the respective combinations of AD-fusion effectors and LacZ reporters were co-transformed into yeast strain EGY48 and transformants were selected and grown on SD/-Trp-Ura dropout media. Yeast transformation and liquid assay were conducted as described in the Yeast Protocols Handbook (Clontech). Yeast β-galactosidase assay were conducted with the Yeast β-Galactosidase Assay Kit (Thermo Scientific). For the yeast two-hybrid assay, the Matchmaker LexA Two-Hybrid System (Clontech) was used. The respective combinations of pLexA and pB42AD fusion plasmids were co-transformed into yeast strain EGY48 containing *p8op-LacZ* plasmid. The empty *pLexA* and *pB42AD* plasmids were co-transformed in parallel as negative controls. Transformants were selected and grown on SD/-His-Trp-Ura dropout plates at 30℃. After 3 d growth, the transformants were grown on SD/-His-Trp-Ura dropout plates containing 80 mg/L X-gal for blue color development.

## **Protoplast Assay**

*Arabidopsis* mesophyll cell protoplasts were prepared and transfected as described previously (16). The promoter-reporter used were the 1580 bp *ELF4* or 946 bp *PRR9* promoters driving firefly luciferase (*pGreen0800II-proELF4:LUC*;

*pGreen0800II-proPRR9:LUC*). The *pGreenII 62-SK-CSU4* and *pGreenII 62-SK-CCA1* were used as the effectors. The dual luciferase kit (Promega) was used for detection of reporter activity. Renilla luciferase driven by a cauliflower mosaic virus 35S promoter was used as an internal control.

## **Quantitative Real-Time PCR**

Total RNA was extracted from 4-d-old *Arabidopsis* seedlings using the RNeasy plant mini kit (QIAGEN). cDNAs were synthesized from 2 µg of total RNA using the 5X All-In-One RT Master Mix cDNA synthesis system (Applied Biological Materials) according to the manufacturer's instructions. Then, cDNAs were subjected to real-time qPCR assays. Quantitative real-time qPCR was performed using the step one plus real-time PCR detection system (Applied Biosystems) and SYBR Green PCR Master Mix (Takara). PCR was performed in triplicate for each sample, and the expression levels were normalized to that of a *PP2A* gene. The primers used in this study were listed in Supplemental Table 1.

## **Isolation of nuclei and cytosol from** *Arabidopsis* **seedlings**

Nuclei and cytosol were extracted with 2 g seedlings grown in darkness using a Plant Nuclei Isolation/Extraction Kit (CelLytic<sup>TM</sup> PN, Sigma-Aldrich). Cell lysis, isolation of nuclei and nuclear protein extraction were performed according to the manufacturer's instructions. After the centrifugation of crude extracts on 1.5 M sucrose solution, the supernatant containing cytosolic, chloroplastic and mitochondrial fractions were stored as the cytosol fraction and the precipitate was used for the nuclear protein extraction.

## **Electrophoretic Mobility Shift Assay (EMSA)**

Bacterial proteins were prepared using induced extracts prepared form *Escherichia coli* transformed with *pET28a-CSU4*, *pCold-TF-CCA1* and *pCold-TF* empty vector. The probes used have been described previously (17, 18); the synthesized

oligonucleotides were annealed, labeled with biotin, and kept in UV light for 30 min for labeling. EMSA was performed using Light Shift Chemiluminescent EMSA kit (Thermo Scientific) as described previously (15). The equal amount of purified His-TF-CCA1, His-CSU4 or His-TF proteins were incubated with 40 fmol biotin-labeled probes in reaction buffer containing 10 mM Tris-HCl pH7.5, 0.05% NP40, 10 mM MgCl<sub>2</sub>, 5% (v/v) glycerol and 0.1  $\mu$ g/mL poly (dI·dC). Reactions were incubated for 20 min at 25℃ and analyzed by separation on 6% nondenaturing polyacrylamide gel, followed by electroblotted to Hybond N+ (Millipore) nylon membranes in 0.5 ×TBE buffer. The detection of labeled probes was performed according to the manufacturer's instructions provided with the EMSA kit.

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**Fig. S1. Map-based cloning of** *CSU4.* (A) Map of the *CSU4* locus and *CSU4* gene structure. The exons are represented by boxes, and the introns are represented by the line. The interval linked to the mutation was sequenced and the base substitution identified. The mutation in *csu4-1* is marked in black with an asterisk. (B) Mutations identified in the *csu4* alleles, and the consequences of mutations to the CSU4 protein. (C) Protein structure of CSU4. DUF167 indicates Domain of Unknown Function 167.



 $csu4-3$  $\overline{\text{Col}}$  $cop1-6$  $csu4-1$  $csu4-2$  $cop1-6$  $cop1-6$  $cop1-6$ 

**Fig. S2.** *csu4* **partially suppress** *cop1-6* **in the light.** Hypocotyl phenotypes and length of 5-d-old Col and three independent *csu4* single mutant seedlings grown in W (14.74  $\mu$ mol/m<sup>2</sup>/s) (A and B), B (3.45  $\mu$ mol/m<sup>2</sup>/s) (C and D), R (129  $\mu$ mol/m<sup>2</sup>/s) (E and F), and FR (4.5  $\mu$ mol/m<sup>2</sup>/s) (G and H) conditions. The unit of hypocotyl length is millimeter (mm). Error bars represent SE ( $n \ge 20$ ). Letters above the bars indicate significant differences ( $P < 0.05$ ) as determined by one-way ANOVA with Tukey's post hoc analysis. The experiments were performed 3 times with similar results. The graphs depict one of three experiments. (I) Phenotypes of Col, *cop1-6*, and three independent *csu4 cop1-6* mutant plants grown in long-day conditions (16 h light/8 h dark) for 35 days.



**Fig. S3. Identification of** *csu4-3* **T-DNA insertion mutant.** (A) Schematic representation of the *CSU4* gene (*At5g63440*). The exons are represented by boxes, and the introns are represented by the line. T indicates the T-DNA insertion position. (B) and (C) RT-PCR and quantitative real-time RT-PCR showing *CSU4* transcript levels in Col and *csu4-3* seedlings grown under long-day conditions for 5 days.



**Fig. S4.** *csu4* **largely suppress** *det1-1* **phenotype.** (A-B) Hypocotyl phenotypes (A) and length (B) of 4-d-old Col, *csu4*, *det1-1* and *csu4 det1-1*seedlings grown in darkness. Error bars represent SE  $(n > 20)$ . Letters above the bars indicate significant differences ( $P < 0.05$ ) as determined by one-way ANOVA with Tukey's post hoc analysis. The experiments were performed 3 times with similar results. The graphs depict one of three experiments. (C) Phenotypes of Col, *det1-1*, and two independent *csu4 det1-1* mutant plants grown in long-day conditions (16 h light/8 h dark) for 45 days.



**Fig. S5. Analysis of** *COP1-6* **mRNA pattern in** *csu4 cop1-6***.** PCR products were generated from Col, *cop1-6* and *csu4 cop1-6* mutant seedlings using primers corresponding to the adjacent exons, and were separated on a 12% acrylamide gel followed by silver staining. M, molecular size markers in base pairs.



**Fig. S6. COP1-SPA complex and DET1 do not regulate CSU4 protein level.** (A) Protein levels of CSU4 in 5-d-old Col, *cop1-4*, *cop1-6*, *det1-1* and various triple *spa* mutants grown in the dark (D), white (W) or Far-red (FR) light as indicated. (B) Protein levels of CSU4 in 5-d-old Col grown in various light conditions (left) or different intensity of white (W) light (right). The unit of light intensity is  $\mu$ mol/m<sup>2</sup>/s<sup>1</sup>. In (A) and (B), *csu4-1* was uses as a negative control. Actin was used as a loading control. (C) Protein levels of COP1 in 5-d-old Col and *csu4* seedlings grown in the D (up) or W light (down). *cop1-6* was uses as a negative control. Actin was used as a loading control. (D) COP1 protein in the cytosol or nucleus of 5 d-old dark grown Col and *csu4* seedlings. *cop1-6* was uses as a negative control. Actin and H3K9ac were used as cytosolic and nuclear markers respectively.



**Fig. S7. CSU4 affects expression of multiple circadian clock genes.** The expression pattern of *TOC1, GI, ELF3, ELF4, LUX, PRR5, PRR7* and *PRR9* transcripts in Col and *csu4* mutant seedlings grown in 12L/12D photoperiods. ZT represents zeitgeber time. Error bars represent SD of three technical replicates. Asterisks represent statistically significant differences ( $*P < 0.05$ ), as determined by Student's t test.



**Fig. S8. The expression pattern of CSU4 mRNA and protein.** (A) The expression pattern of *CSU4* transcripts in Col seedlings grown in 12L/12D photoperiods. *CCA1* served as a control. ZT represents zeitgeber time. Error bars represent SD of three technical replicates. (B) The expression pattern of *CSU4* protein in 5-d-old Col seedlings grown in a 24 h period (12h dark/12h light) as indicated time points. *csu4-1*  was used as a negative control. ZT represents zeitgeber time. Actin served as a loading control.



**Fig. S9. CCA1 is independent of CSU4 with respect to hypocotyl growth in W, B, R and FR light conditions.** Hypocotyl phenotypes and length of 5-d-old Col, *csu4-1,*   $ccal-22$  and  $csu4-1$   $ccal-22$  mutant seedlings grown in W (14.74  $\mu$ mol/m<sup>2</sup>/s) (A and B), B (3.45  $\mu$ mol/m<sup>2</sup>/s) (C and D), R (129  $\mu$ mol/m<sup>2</sup>/s) (E and F), and FR (4.5  $\mu$ mol/m<sup>2</sup>/s) (G and H) conditions. The unit of hypocotyl length is millimeter (mm). Error bars represent SE ( $n \geq 20$ ). Letters above the bars indicate significant differences ( $P < 0.05$ ) as determined by one-way ANOVA with Tukey's post hoc analysis. The experiments were performed 3 times with similar results. The graphs depict one of three experiments.

Primer's name	Primer sequences $(5^3 \rightarrow 3^3)$	
<b>Plasmid Constructs</b>	Underscored nucleotides indicate restriction	
	sites for cloning	<b>Construct's name</b>
	GGGGACAAGTTTGTACAAAAAAGCAGGCTT	pDONR221-CSU4
CSU4attB1	CATGCCGAAGAGAACGACACAC	
CSU4attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC	
	CGGTACAACGGCCTCGAGAAG	
CCA1attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTT	pDONR223-CCA1
	CATGGAGACAAATTCGTCTGGAGAAG	
CCA1attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC	
	TGTGGAAGCTTGAGTTTCCAAC	
CSU4pro-CSU4-Eco	CCGGAATTCGTAATGTGATTTAGATGACAT	$pCambia$ 1300-pro $C$ $SU4$ :CSU4
RI(F)	<b>TATC</b>	
CSU4pro-CSU4(R)	CATGCCATGGGGAATCTAAAGAGTATAGAG	
	$\mathcal{C}$	
$CSU4-EcoRI(F)$	TTCGCAACGGCGACTGGCTGGAATTCATGC	pLexA-CSU4
	CGAAGAGAACGACAC	
$CSU4-BamHI(R)$	GAGCGGCCGCCATGGTCGACGGATCCTTAC	
	GGTACAACGGCCTCG	
CSU4-N-EcoRI(F)	CTGGAATTCATGCCGAAGAGAACGACAC	pLexA-CSU4 N
CSU4-N-BamHI(R)	GACGGATCCTTATCCACCGTCGAGTTGTGA	
	<b>AATG</b>	
$CSU4-C-Gibson(F)$	TTCGCAACGGCGACTGGCTGGAATTCCTTG	pLexA-CSU4 C
	TTCAGGTTGCAATAGAAG	
$CSU4-C-BamHI(R)$	GAGCGGCCGCCATGGTCGACGGATCCTTAC	
	GGTACAACGGCCTCG	
$CSU4-EcoRI(F)$	TGCCAGATTATGCCTCTCCCGAATTCATGCC	pB42AD-CSU4
	GAAGAGAACGACAC	
$CSU4-Xhol(R)$	GGCGAAGAAGTCCAAAGCTTCTCGAGTTAC	
	GGTACAACGGCCTCGAGAAG	
$CCA1-EcoRI(F)$	CCCGAATTCATGGAGACAAATTCGTCTGGA	pB42AD-CCA1
	GAAG	
$CCA1-Xhol(R)$	CTTCTCGAGTCATGTGGAAGCTTGAGTTTCC	
	<b>AAC</b>	
ELF4pro-KpnI(F)	CTCGGTACCCTGCACCGTGGCAAGTCGTAC	pLacZi2u-ELF4p
ELF4pro-XhoI(R)	TGCCTCGAGAATAATTTTTAATTGTGTTTTT	
	<b>CTCTC</b>	
$CSU4-EcoRI(F)$	GTGGATCCCCCGGGCTGCAGGAATTCATGC	pGreenII 62-SK-CSU4
	CGAAGAGAACGACAC	
$CSU4-Xhol(R)$	GAATTGGTACCGGGCCCCCCCCTCGAGTTAC	
	GGTACAACGGCCTCG	

**Supplemental Table 1. Primers used in this study**



