Ogiso et al.
Supplemental data
This section contains the legends to Supplementary Figures 1 to 10, and Supplemental Tables 1 to 4

Supplemental figures

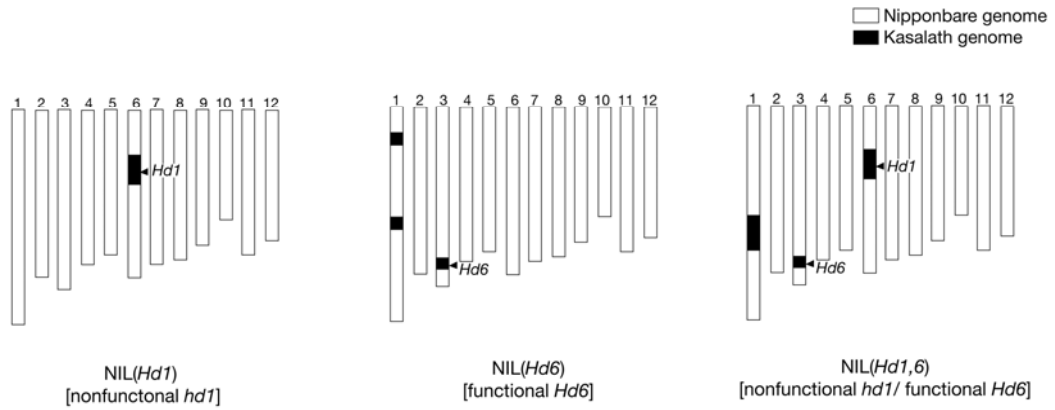


Figure S1. Graphical genotypes of NILs.
Black and white regions represent segments of the 12 chromosomes derived from Kasalath and Nipponbare, respectively. The Kasalath allele of *Hd6* is functional in NIL(*Hd6*) and NIL(*Hd1*,*6*). The Nipponbare allele of *Hd1* is functional in NIL(*Hd6*) and Nipponbare. DNA markers for selection of Kasalath fragments for the *Hd1* and *Hd6* regions were R2171 to R2654 and R2311 to C217, respectively.

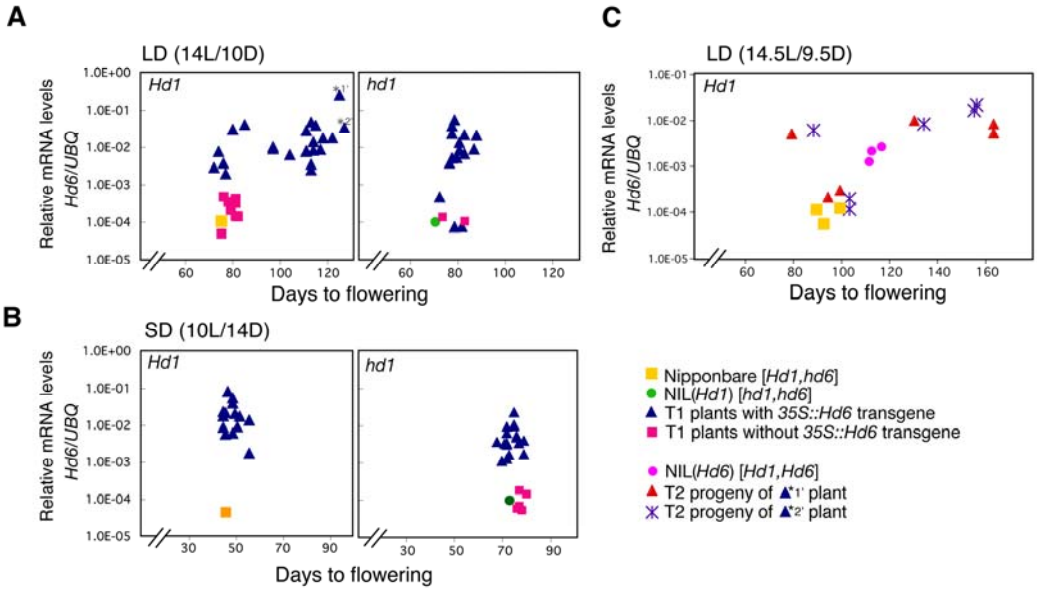


Figure S2. Functional *Hd1* gene is necessary for the late flowering observed in *Hd6*-overexpressing lines under LD conditions.
(A–C) Flowering time (x-axis) vs. *Hd6/UBQ* expression levels (y- axis) of transgenic lines in the T1 and T2 generations. Transgenic lines were generated in two genetic

backgrounds: Nipponbare (with a functional *Hd1* allele) and NIL(*Hd1*) (an NIL with a Kasalath *Hd1* allele [a non-functional *Hd1* allele]). T1 and control plants were grown under LD (A) and SD (B) conditions. *Hd6* dose-dependency of days to flowering was observed only with the functional *Hd1* allele. (C) Flowering times of T2 progeny plants from T1 plants (*1, *2) under LD conditions.

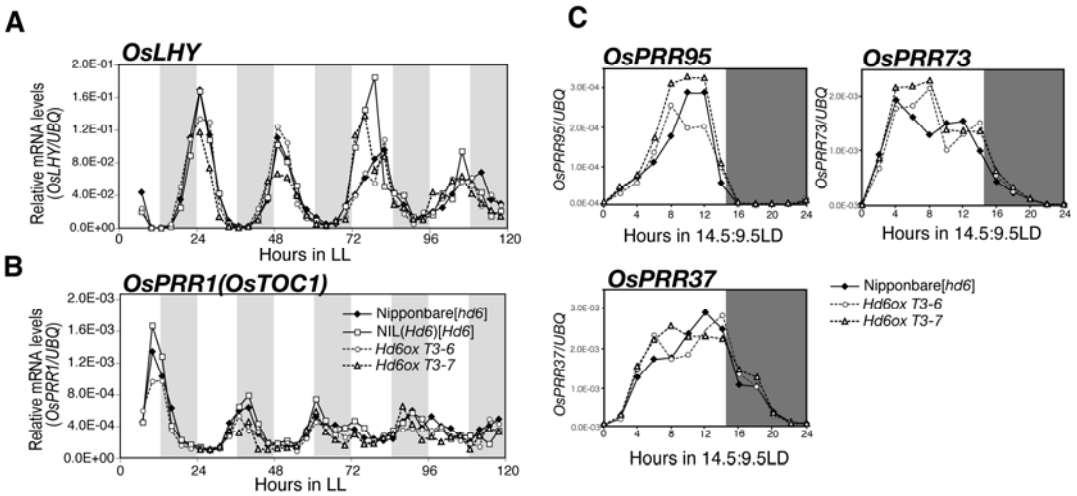


Figure S3. *Hd6* ox does not affect the rhythms of clock-controlled gene expression in continuous light (LL).

(A)(B) *OsLHY* (A) and *OsPRR1* (B) mRNA expression in Nipponbare [*Hd1+/hd6-*], NIL(*Hd6*) [*Hd1+/Hd6+*], and transgenic rice plants overexpressing *Hd6* cDNA [*Hd1+/Hd6ox*] was examined under LL conditions. Seedlings were grown under 12L/12D conditions for 10 days and then transferred to LL conditions; sample seedlings were collected every 3 h. Each point represents the average of three biological repeats. The values for each biological repeat consist of three repeated measurements. (C) *OsPRR 95, 73*, and *37* mRNA expression in Nipponbare [*Hd1+/hd6-*] and in transgenic rice plants overexpressing Hd6 cDNA [*Hd1+/ Hd6ox*] was examined under LD conditions.

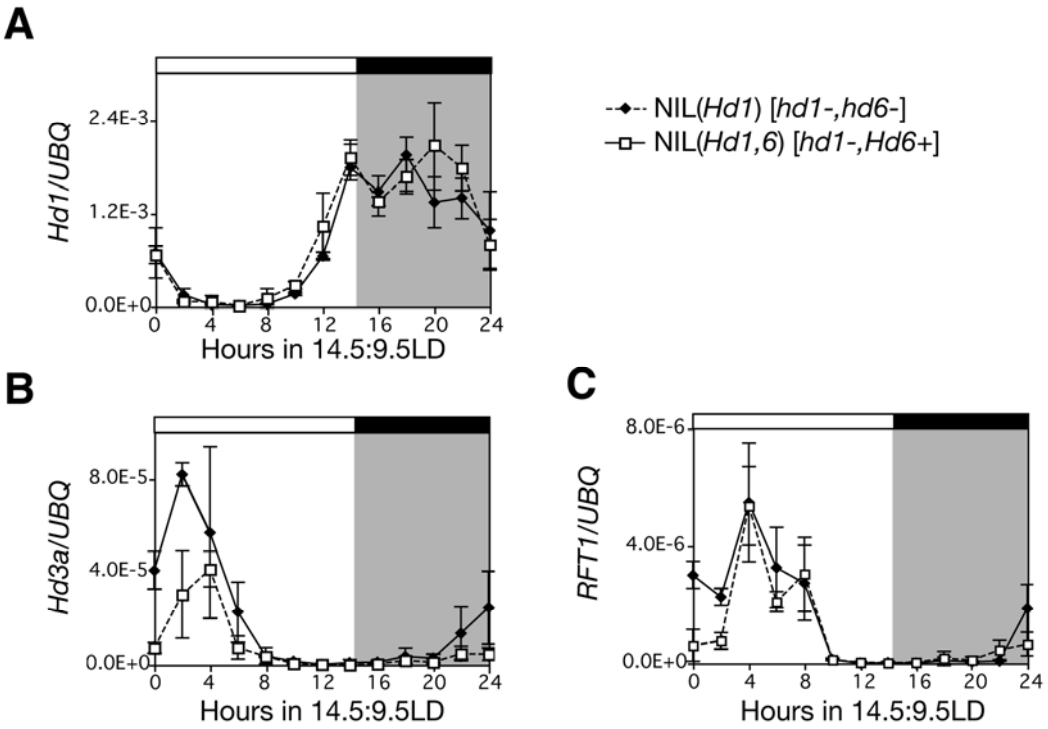


Figure S4. *Hd6* does not repress *Hd3a* and *RFT1* critically in an *hd1* non-functional background.

(A)–(C) Plants were grown under LD (14L/10D) conditions for 30 days. *Hd1* (A), *Hd3a* (B), and *RFT1* (C) mRNA expression is shown for NIL(*Hd6*) [*Hd1+/ Hd6+*] and NIL(*Hd1,6*) [*hd1-/ Hd6+*]. Each point represents the average of three biological repeats. Each average was obtained from three independent measurements. Error bars are S.D.

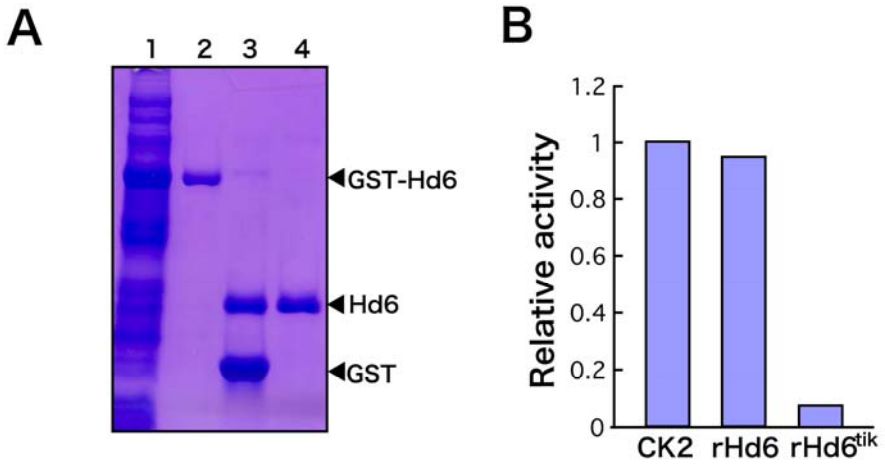


Figure S5. Activity of purified recombinant Hd6 and Hd6^{tik} proteins expressed in *E. coli*

(A) SDS-PAGE analysis of purified recombinant Hd6. The GST-fused Hd6 was expressed in *E. coli* and subjected to affinity purification using glutathione sepharose beads (lane 2). The beads were washed and subjected to thrombin cleavage as described (lane 3). The supernatant containing the purified protein was concentrated and analyzed

by SDS-PAGE (lane 4). Lane 1 shows the crude extract. (B) Recombinant Hd6 purified from *E. coli* had CK2 protein kinase activity, but recombinant Hd6^{tik} had reduced activity. CK2 activity was determined by the incorporation of ^{32}P phosphate into the CK2 substrate peptide (RRRDDDSDDD) (Upstate Biotechnologies). Human CK2 alpha (Biaffin GMBH & CO KG) is normalized to 1.

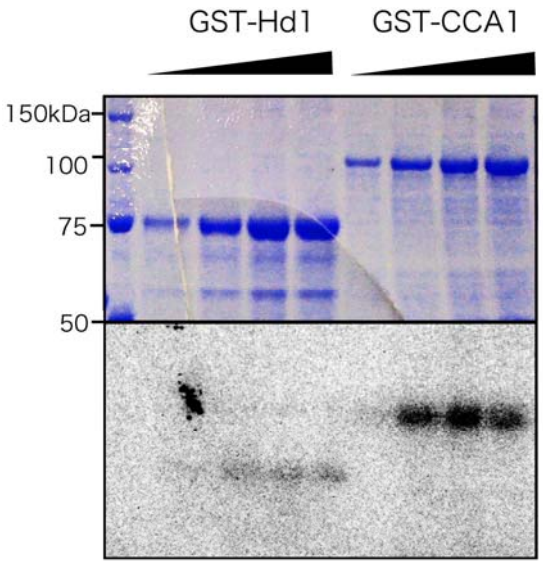


Figure S6. CK2(Hd6) phosphorylation assay for Hd1.
SDS-PAGE and autoradiographs of SDS-PAGE analysis of GST-Hd1 and GST-CCA1 after *in vitro* CK2(rHd6) phosphorylation assays. Hd1 protein was not a target substrate protein for Hd6 CK2 kinase *in vitro*.

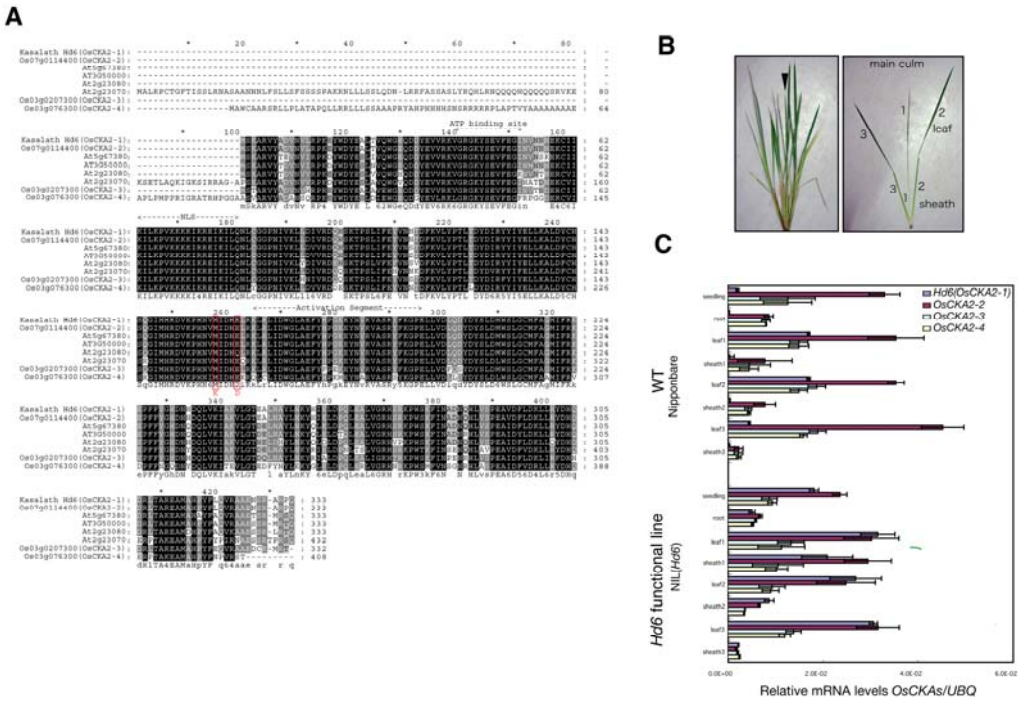


Figure S7. CK2α genes in rice.

Protein sequence alignment of all of CK2α subunit genes in rice and the Arabidopsis genome. Functional domains conserved in CK2α are indicated by arrows: ATP binding site, basic stretch (NLS), catalytic loop and activation segment. For Hd6 (OsCKA1) the Kasalath functional allele is presented (accession no. BAB21591), since the reference Nipponbare genome contains the mutated Hd6 gene. Red boxes indicate timekeeper mutation points. (B) WT plant 40 days after germination, grown under LD conditions (14.5L/9.5D). Black arrow shows main culm. Right panel shows parts sampled for expression analysis in rice main culm. (C) Expression analysis of all four rice CK2α genes (OsCKAs). Seedlings were grown in LD cycles. Samples for mRNA extraction were collected 10 days (whole plant and root) or 40 days (meristem, leaf, and leaf sheath) after sowing. Expression of OsCKAs was not detected in meristems. Expression was examined by TaqMan real-time RT-PCR (relative to ubiquitin expression). In Nipponbare, Hd6 mRNA expression was likely to have been reduced by non-sense-mediated mRNA decay.

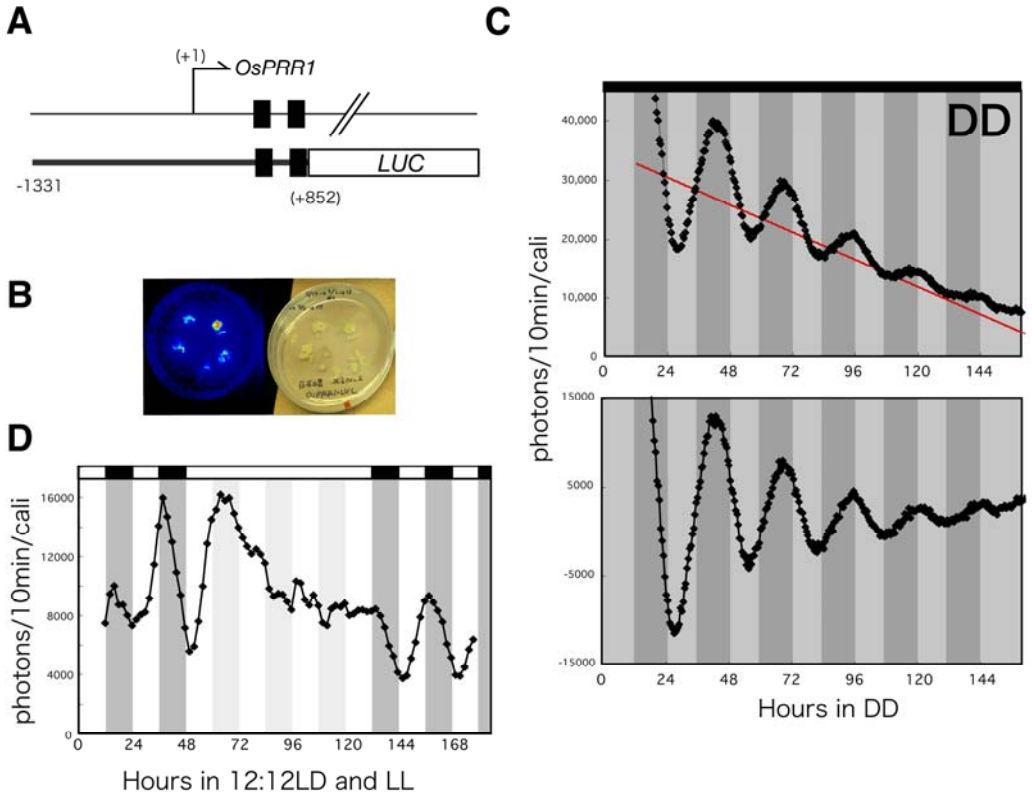


Figure S8. Circadian clock monitoring by using *OsPRR1:luc* in rice cells.
(A) Schematic of the construct used in this experiment. (B) Bioluminescent images of transformed rice calli. Transformed calli were deposited in a 3-cm Petri dish for bioluminescence measurement. (C) Calibration of rhythmic bioluminescence data. Raw bioluminescence data (top panel) were calibrated by simple subtraction after the addition of a line (in red); calibrated data are shown in the bottom panel. (D) Arrhythmic expression of *OsPRR1:luc* in LL. Rhythmic expression of *OsPRR1:luc* recovered in the LD cycles after LL treatment.

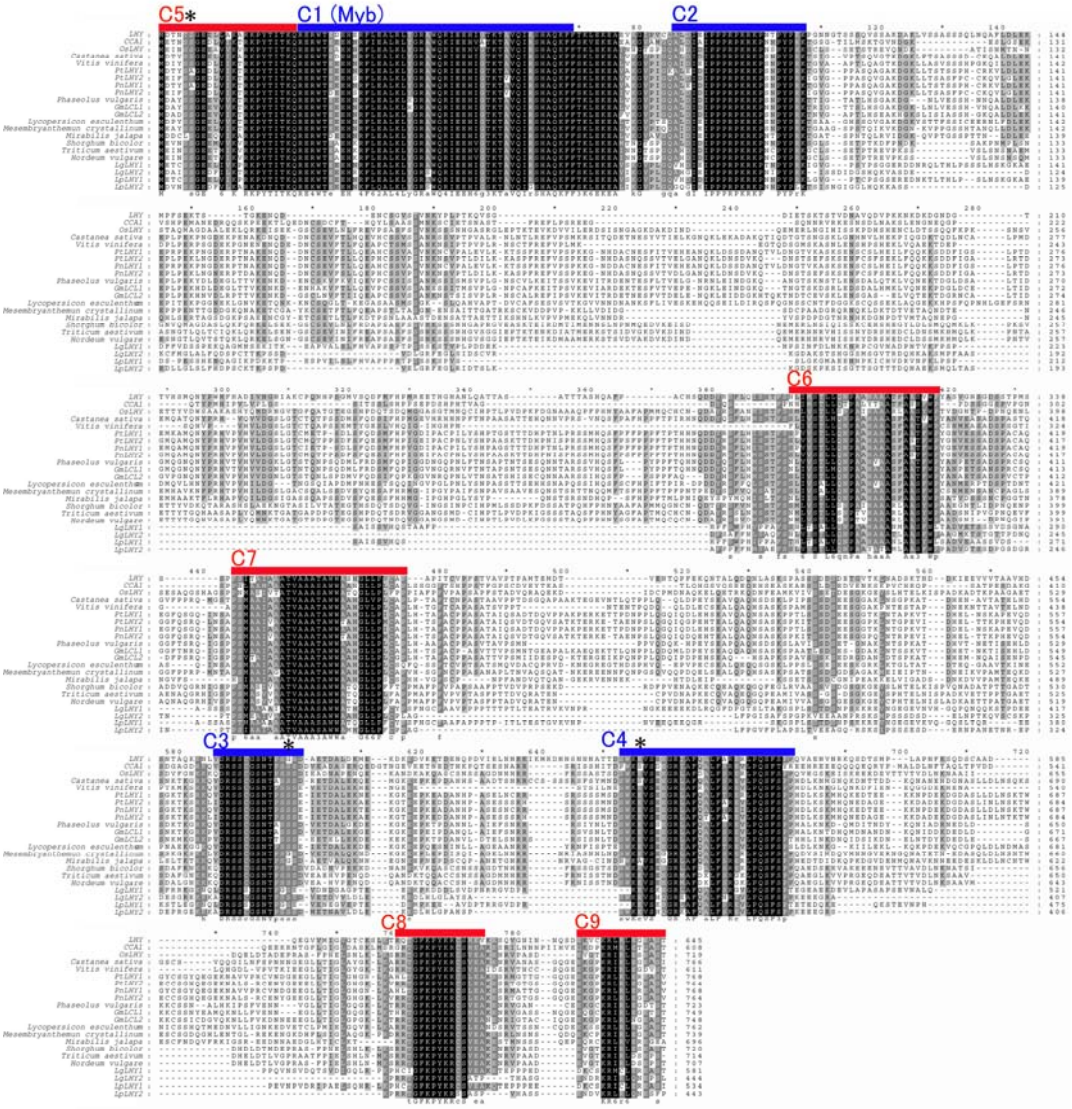


Figure S9. Multiple alignment of LHY, CCA1, OsLHY, and LHY-like proteins. (A) Species (clones) used in this alignment were *Arabidopsis thaliana* (LHY; At1g01060, CCA1; At2g46830),*Oryza sativa* (OsLHY; Os08g0157600), *Castanea sativa* (AAU20773), *Vitis vinifera* (GSVIVT00026185001), *Populus trichocarpa* (PtLHY1;NC_008468, PtLHY2;NC_008480), *Populus nigra* (PnLHY1;AB429410, PnLHY2;AB429411), *Phaseolus vulgaris* (CAD12767), *Glycine max* (GmLCL1;ABW87008, GmLCL2;ABW87009), *Lycopersicon esculentum* (BT012912), *Mesembryanthemum crystallinum* (AAQ73524.1), *Mirabilis jalapa* (ACL81163), *Sorghum bicolor* (Sb07g003870), *Triticum aestivum* (EST: dbj|CJ718789.1| CJ720523.1| CJ717710.1| CJ615885.1| CJ614670.1| CJ613519.1|), *Hordeum vulgare* (EST : HO13M22S, AV933133, AV934889, AV937857, AV909868, AV941397), *Lemna gibba* (LgLHY1; BAD97870, LgLHY2;BAD97871), and *Lemna paucicostata* (LpLHY1; BAD97866, LpLHY2; BAD97867) . Conserved (black), similar (grey) and non-conserved amino acid residues (white) were highlighted with GENEDOC software using amino acid sequences predicted from the full-length cDNA sequences in public

databases. Red bars indicate domains conserved in land plants (Okada et al., 2009). Blue bars indicate domains conserved named C5-C9 in angiosperms. Asterisks indicate CK2 phosphorylation sites in CCA1.

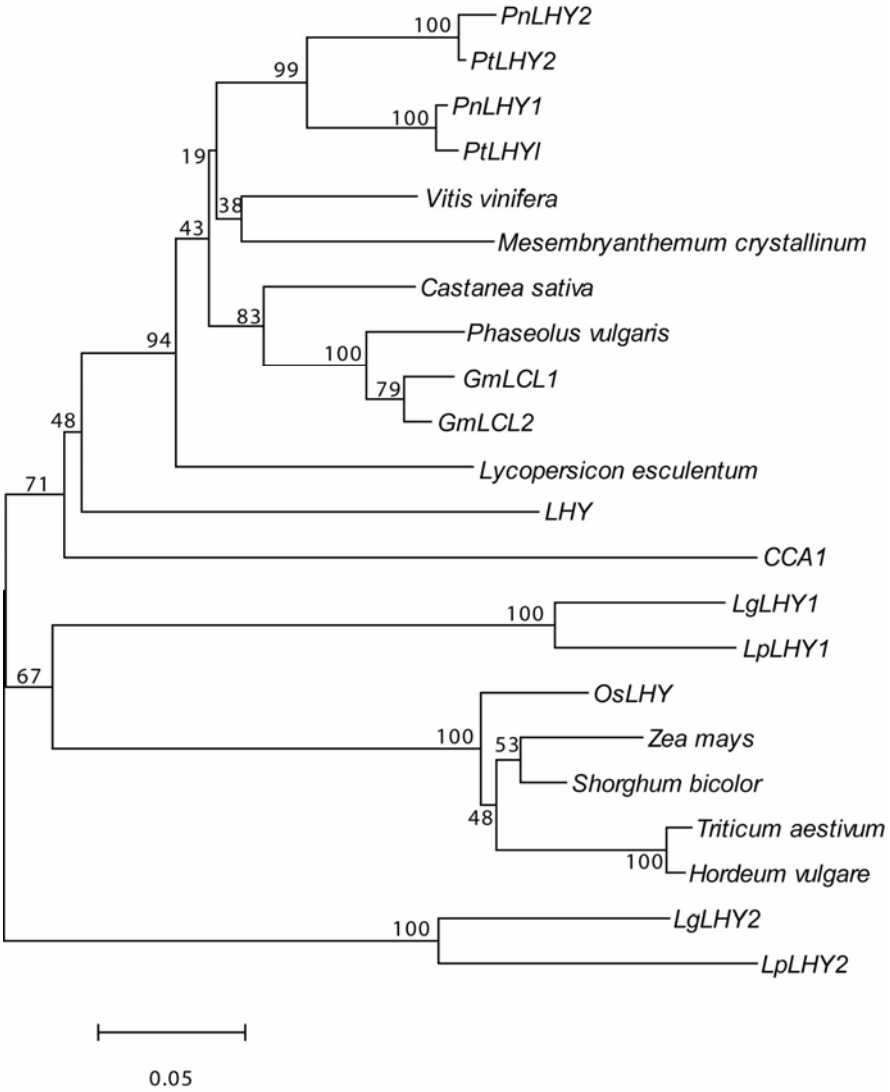


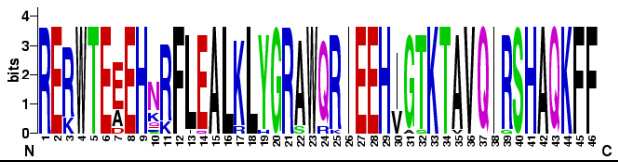
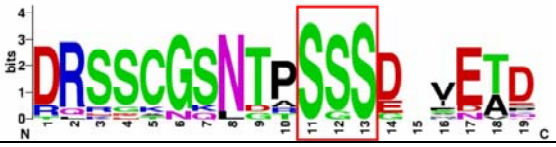
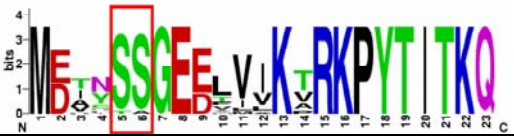
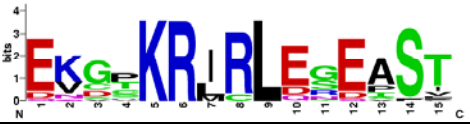
Figure S10 Evolutionary relationships of 22 taxa in the angiosperm CCA1/LHY-like gene.

Evolutionary history was inferred by using the neighbor-joining method [1]. The optimum tree with the sum of branch length = 2.01178381 is shown. The percentages of replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed by using the Poisson correction method [3] and are in units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 2.25). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 274 positions (extracted

C1–5 domain) in the final dataset. Phylogenetic analyses were conducted in MEGA4 [4].

Supplementary Tables

Supplementary Table 1 C1 to C9 domains and conserved CCA1 phosphorylation sites from LHY/CCA1-like proteins in angiosperms.

domain name	function	Sequence
C1	Myb domain	
C2	unknown	
C3	unknown	
C4	CCA1 homodimerization domain	
C5	unknown	
C6	unknown	
C7	unknown	
C8	unknown	
C9	unknown	

C1~4 domain : common domains of LHY/CCA1 in land plant
C5~9 domain common domains of LHY/CCA1 in Angiosperm
Red boxes indicate CCA1 phosphorylation sites.

Supplementary Table 2 C4 domain-like sequences in database

Gene name	Species	Classification	Accession number/geme ID
CCA1	*Arabidopsis*	Eudicots/eurosids II	At2g46830
LHY			At1g01060
	Brassica oleracea	Eudicots/eurosids II	AM390288
	Gossypium hirsutum	Eudicots/eurosids II	DW511768
	Poncirus trifoliata	Eudicots/eurosids II	EY813527
	Citrus aurantium	Eudicots/eurosids II	EY842696
	Citru sinensis	Eudicots/eurosids II	EY655215
	Carica papaya	Eudicots/eurosids II	EX259210
	Eucalyptus globulus	Eudicots/eurosids I	ES594501
	Vitis vinifera	Eudicots/rosids	GSVIVT00026185001
	Fragaria vesca	Eudicots/eurosids I	DY668428
	Prunus persica	Eudicots/eurosids I	BU048428
		Eudicots/eurosids I	DY640705
	Malus domestica	Eudicots/eurosids I	GO497876
	Castanea sativa	Eudicots/eurosids I	AY611029
	Cucumis melo	Eudicots/eurosids I	AM725612
	Pisum sativum	Eudicots/eurosids I	AY826730
GmLCL1	*Glycine max*	Eudicots/eurosids I	EU076433
GmLCL2			EU076434
	Medicago truncatula	Eudicots/eurosids I	BI311517
	Phaseolus vulgaris	Eudicots/eurosids I	AJ420902
	Populus euphratica	Eudicots/eurosids I	AJ768814
PtLHY1	*Populus trichocarpa*	Eudicots/eurosids I	*(Takata el al., 2009)
PtLHY2			CV238604
PnLHY1	*Populus nigra*	Eudicots/eurosids I	AB429410
PnLHY2			AB429411
	Euphorbia esula	Eudicots/eurosids I	DV129915
	Manihot esculenta	Eudicots/eurosids I	CK643975
	Ricinus communis	Eudicots/eurosids I	EG658583
	Mesembryanthemum crystallinum	Eudicots/core Eudicots	AY371287
	Aquilegia formosa x A.pubescens	Eudicots	DR935954
	Nicotiana benthamiana	Eudicots/euasterid I	EC917354
	Nicotiana tabacum	Eudicots/euasterid I	EB450103
	Lycopersicon esculentum	Eudicots/euasterid I	AI776709
	Mentha piperita	Eudicots/asterids	AW254718
	Coffea canephora	Eudicots/euasterid I	DV690965
	Helianthus argophyllus	Eudicots/euasterid II	EE611452
	Liriodendron tulipifera	MAGNLLIIDS	DT581324
	Persea americana	MAGNLLIIDS	FD503075
	Aristolochia fimbriata	MAGNLLIIDS	FD750795
	Asparagus officinalis	Monocots	CV291009
OsLHY	*Oryza sativa*	Monocots	Os08g0157 600
	Cenchrus ciliaris	Monocots	EB658854
	Lolium multiflorum	Monocots	AU247776
	Hordeum vulgare	Monocots	CD662835
	Triticum aestivum	Monocots	CJ718789
	Agrostis capillaris	Monocots	DV854668
	Saccharum officinarum	Monocots	CA242688
	sorghum bicolor	Monocots	Sb07g00387 0
	Panicum virgatum	Monocots	FE628858
	Zea mays	Monocots	CF058782
	Zingiber officinale	Monocots	DY347440
	Musa acuminata	Monocots	DN239789
	Elaeis guineensis	Monocots	EL682537
LgLHY1	*Lemna gibba*	Monocots	BAD97870
LgLHY2		Monocots	BAD97871
LpLHY1	*Lemna paucicostata*	Monocots	BAD97866
LpLHY2		Monocots	BAD97867
	Pinus pinaster	Pinophyta	CT577466
	Pinus taeda	Pinophyta	CF477745
	Selaginella moellendorffii	Pteridophyta	FE499712
PpCCA1a	*Physcomitrella patens*	Bryophyta	AB458831
PpCCA1b			AB458832

Supplementary Table 3 Primers used for constructs in this work

Primer name	Sequence
Hd6–1F	5’-ATGTCGAAGGCGAGGGTCTA-3’
Hd6-tik-F2	5’-ACAATGTTAAAATAGATCATGACCTCCGAA-3’
Hd6-R	5’-TTCGGAGGTCATGATCTATTTTAACATTGT-3’
Hd6-tik-R2	5’-TCATTGTGGTCGTGCTCTGCTA-3’
FlagNF1	5’-CTCCGATTACAAGGATCATGATGGAGATTACAAGGATCATGATATTGATT-3’
FlagNF2	5’-AAAGATCTTAATTAAAGCAACCATGGCTTCCTCCGATTACAA-3’
FlagN1R1	5’-CGGGGGATCCCTTATCATCATCATCCTTGTAATCAATATCATGATCC-3’
FlagN1R2	5’-TTGAGCTCCGGCGCGCCACTAGTGGGGTACCCCCGGGGGATCCCT-3’
FlagN2R1	5’-CGGGGGATCCCCTTATCATCATCATCCTTGTAATCAATATCATGATCC-3’
FlagN2R2	5’-TTGAGCTCCGGCGCCCACTAGTGGGGTACCCCCGGGGGATCCCCT-3’
FlagN3R1	5’-CGGGGGATCCTTATCATCATCATCCTTGTAATCAATATCATGATCC-3’
FlagN3R2	5’-TTGAGCTCCGGCGCGCCACTAGTGGGGTACCCCCGGGGGATCCT-3’
XbaI-strepII-BamHI-F	5’-CGCGCCTTTTTCGAACTGCGGGTGGCTCCAGCTAGCCATTCTAG-3’
XbaI-strepII-BamHI-R	5’-AATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGGCGCGGATC-3’
OsLHY-Sal-F	5’-AAGTCGACTTGTTATATTTCTTCTTTTTCTTGAA-3’
CCA1-SalI-F	5’-AAGGTCGACAAGAGGAGCTTAGTGATGGA-3’
CCA1-R	5’-TGTAGCAGTGGTCTTGAAAAC-3’
CCA1-S>A-F	5’-TCCATGGAAGGCTGTGTCTGA-3’
CCA1-S>A-R	5’-ACACAGCCTTCCATGGATCGGTTATATT-3’
CCA1-S>E-F	5’-TCCATGGAAGGAAGTGTCTGA-3’
CCA1-S>E-R	5’-ACACTTCCTTCCATGGATCGGTTATATT-3’
CCA1-56 7-S>A-F	5’-GTCGACATGGAGACAAATGCGGCTGGAG-3’
CCA1-S>AAA-F	5’-CACTCCGGCGGCTGCTGATGATGTTGAGGCGGAT-3’
CCA1-S>AAA-R	5’-CAGCAGCCGCCGGAGTGTTTGAGCCACAC-3’
KpnI-OsPRR1pro-F	5’-AAAAAAGGTACCATCGCTAGAACAAGGGTCAC-3’
HindIII-OsPRR1ex2-R	5’-AAAAAGCTTTGCGATGTATTTGAGCATCTT-3’
mOsLHY-5,6S>A-F	5’-AAAAAAGTCGACTTGGGAATGGAGATTAATGCCGCTGGTG-3’
mOsLHY-SSS-F	5’-GTTCCAACACACCGGCAGCTGCTGATATAGAAGCAGATA-3’
mOsLHY-SSS-R	5’-TCTGCTTCTATATCAGCAGCTGCCGGTGTGTTGGAACCA-3’
mOsLHY-600E>A-F	5’-TGATTCATGGAAGGCAGTTTCTGAAGA-3’
mOsLHY-600E>A-R	5’-TCTTCAGAAACGCTCTTCCATGAATCA-3’
OsLHY-NotI-R	5’-TTGCGGCCGCATCATGTCGATGCTTCGCT-3’
OsLHY-SpeI-R	5’-GGAACTAGTCCATCATGTCGATGCTTCGCT-3’
OsLHY-stopX-XhoI-R	5’-TTTCTCGAGCCATGTCGATGCTTCGCT-3’
XhoI-3FLAG-F	5’-TCGAGGGAGGTGGAGATTACAAGGATCATGATGGAGATTAT-3’
XhoI-3FLAG-R	5’-CTTTATAATCTCCATCATGATCCTTGTAATCTCCACCTCCC-3’
NotI-3FLAG-F	5’-AAAGATCATGATATTGATTACAAGGATGATGATGATAAGTAGC-3’
NotI-3FLAG-R	5’-GCTACTTATCATCATCATCCTTGTAATCAATATCATGATCTTT-3’

Supplementary Table 4 Primers and Taq-man probes for quantitative RT-PCR

OsLHY-1828F	5’-GGGTCGTCTGGCTTTTGAT-3’
OsLHY-2101R	5’-CGGTACCCTGTTCTCCTTC-3’
OsLHY probe	5’-AAAGGAGATTAGCAAGGAGGAAGAAG-3’
OsPRR1-56F	5’-ACCCATGTGTGGCGGC-3’
OsPRR1-129R	5’-GCCAACTCGAAATTGTCATTGAA-3’
OsPRR1 probe	5’-CGGATGCTTGGTTTGTCGGAGAAAAA-3’
OsGI-2793F	5’-GCATAAGTTGTGGGTGCTTCC-3’
OsGI-2842R	5’-GAAAATACGCAGCTGGTGGAG-3’
OsGI-2870T probe	5’-AGATCCTCGGCTGTAAGTTGTTGGAGGC-3’
RFT1-418F	5’-CGTCCATGGTGACCCAACA-3’
RFT1-501R	5’-CCGGGTCTACCATCACGAGT-3’
RFT1-452T probe	5’-CGGTGGCAATGACATGAGGACGTTC-3’
Hd6(OsCKA2-1)-1193F	5’-TCACCAAGATAGGCTCACTGCA-3’
Hd6(OsCKA2-1)-1343R	5’-CAAAGTAGTACCGTCGTGGATCAT-3’
Hd6(OsCKA2-1)-T probe	5’-TGGCACATCCGTACTTCCTCCAAGTGAG-3’
OsCKA2-2-1021F	5’-GGAAGGCACAGCAGGAAAC-3’
OsCKA2-2-1247R	5’-TCCATGACTGCTGCTAATCG-3’
OsCKA2-2 probe	5’-AGAACAGTAGGCCTCGTGCACAATAGACAAAAG-3’
OsCKA2-3-F	5’-CCACCATGACAGGCTGACC-3’
OsCKA2-3-R	5’-TGCTGCCCTCACTTGTTCAA-3’
OsCKA2-3 probe	5’-CTCGTGAAGCTATGGCGCATCCCTAC-3’
OsCKA2-4-1164F	5’-GGAAAGGCCTACAGCAAAGGA-3’
OsCKA2-4-1237R	5’-TCCTGCCGTTTTAAGTGCTTC-3’
OsCKA2-4-1187T probe	5’-CCATGGCTCATCCATATTTCAATCCAGTG-3’
OsPRR73-F	5’-GAGCAGTTGACTTTCTAGTGAAGCCT-3’
OsPRR73-R	5’-TTCGGATGCCACTTTCGCT-3’
OsPRR73-Probe	5’-AGCATGTTTGGAGACGATGCCACAGTTAA-3’
OsPRR95-F	5’-GCTGCAGATTTCCTTGTTAAGCC-3’
OsPRR95-R	5’-TGCTGCACATCAAGAACACCG-3’
OsPRR95-Probe	5’-TGGCAGCATGTTTGGAGAAAACAACTGTCC-3’
OsPRR37-F	5’-CAGAAAAGGAAAGAGCGCAAC-3’
OsPRR37-R	5’-CTGCTCGGCCAGCCTC-3’
OsPRR37-Probe	5’-TCGGAAAAAAGGTGCGGTACCAGAG-3’

1. **Saitou N & Nei M** (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4:**406–425.

2. **Felsenstein J** (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39:**783–791.

3. **Zuckerkandl E & Pauling L** (1965) Evolutionary divergence and convergence in proteins, pp. 97–166 in Evolving Genes and Proteins, edited by V. Bryson and H.J. Vogel. Academic Press, New York.

4. **Tamura K, Dudley J, Nei M & Kumar S** (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution **24:**1596–1599.

5. **Crooks GE, Hon G, Chandonia JM, Brenner SE** (2004) WebLogo: A sequence logo generator. Genome Research**14:**1188–1190.