Casein kinase II (CK2) is a protein kinase with an evolutionarily conserved function as a circadian clock component in several organisms, including the long-day plant Arabidopsis (Arabidopsis thaliana). The circadian clock component CIRCADIAN CLOCK ASSOCIATED1 (CCA1) is a CK2 target in Arabidopsis, where it influences photoperiodic flowering. In rice (Oryza sativa), a short-day plant, Heading date6 (Hd6) encodes a CK2α subunit that delays flowering time under long-day conditions. Here, we demonstrate that control of flowering time in rice by the Hd6 CK2α subunit requires a functional Hd1 gene (an Arabidopsis CONSTANS ortholog) and is independent of the circadian clock mechanism. Our findings from overexpressing the dominant-negative CK2 allele in rice support the independence of CK2 function from the circadian clock. This lack of control of the circadian clock by Hd6 CK2α might be due to the presence of glutamate in OsLHY (a CCA1 ortholog in rice) instead of the serine at the corresponding CK2 target site in CCA1. However, this glutamate is critical for the control of the OsPRR1 gene (a rice ortholog of the Arabidopsis TOC1/PRR1 gene) by OsLHY for regulation of the circadian clock. We also demonstrated that the other conserved CK2 target sites in OsLHY conferred robust rhythmic expression of OsLHY-LUC under diurnal conditions. These findings imply that the role of CK2 in flowering-time regulation in higher plants has diversified during evolution.

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proteins, including transcription factors, and can modulate DNA-binding ability, intracellular localization, and protein stability and by interacting with partner proteins in insects and animals (Miyata and Nishida, 2004; Riera et al., 2004; Krick et al., 2006; Pagano et al., 2006; Taghli-Lamallem et al., 2008). Consistent with this, a study in antisense CK2α lines has suggested the pleiotropic functions of CK2 in Arabidopsis (Lee et al., 1999). Recently, a dominant-negative form of CK2α was introduced into Arabidopsis to further confirm the pleiotropic functions of CK2 (Moreno-Romero et al., 2008).

In rice (Oryza sativa), a SD plant, we previously used natural variations among rice cultivars to identify the Hd6 gene as encoding an α-subunit of CK2 (Takahashi et al., 2001). Hd6 functional lines show late flowering under natural and long-day (LD) conditions. However, the molecular mechanism behind the promotion of late flowering by Hd6 has not been elucidated. In this paper, we analyzed the function of Hd6 in the rice circadian clock and in the photoperiodic flowering pathway. We revealed that the circadian clock has been disconnected from the function of CK2 in the photoperiodic control of flowering in rice, partly because of the presence of an evolutionarily conserved Glu site in OsLHY in rice, of which the corresponding site is Ser (a CK2 target site) in CCA1.

RESULTS

Hd6 Requires Functional Hd1 for Flowering Repression under LD Conditions

To elucidate the molecular function of Hd6, we examined flowering time in rice with functional, non-functional, or overexpressed Hd6 alleles (including nearly isogenic lines and transgenic lines) under LD and SD conditions (Fig. 1; Supplemental Fig. S1). Under LD conditions, Hd6 delayed flowering time in a dose-dependent manner (Fig. 1; Supplemental Fig. S2). This Hd6-induced delay required the presence of a functional Hd1 allele. A 30-min extension of the LD photoperiod caused a synergistic delay in flowering through the interaction of Hd6 with Hd1; therefore, Hd6 can enhance Hd1 floral repression activity under LD conditions. The time of flowering under SD conditions was also examined in plants with these Hd6-related alleles; the results suggested that Hd6 plays a critical role in Hd1 activity (Fig. 1; Supplemental Fig. S2).

Hd6 Does Not Affect Circadian Rhythms in Rice

Because we found genes orthologous to the circadian clock genes of Arabidopsis in the rice genome (Izawa, 2007b), we used Hd6 alleles to examine the expression of circadian clock-controlled genes in rice. Under constant light or darkness, cab1R:LUCIFERASE (LUC) expression (Sugiyama et al., 2001) did not change in plants with these Hd6 alleles (Fig. 2, A–D). Expression of OsLHY and OsPRR1 (a TOC1 ortholog; Murakami et al., 2003, 2007) was also not affected under constant light (Supplemental Fig. S3, A and B). Furthermore, diurnal expression of OsLHY, OsPRR1, -95, -73, and -37, and OsGI (a GIGANTEA ortholog) did not change under LD conditions (Fig. 2, F–H; Supplemental Fig. S3C). These results clearly indicated that Hd6 is not a circadian clock component in rice. Therefore, Hd6 regulation of flowering time is not mediated by changes in circadian clock action.

Hd6 Delays Flowering by Repressing FT-Like Genes under LD Conditions

We next analyzed the expression patterns of flowering-time genes in Nipponbare, a nearly isogenic line of Hd6 [NIL(Hd6); Supplemental Fig. S1], and in lines overexpressing Hd6 under LD conditions. Consistent with the delayed flowering phenotype, the overexpression of Hd6 repressed expression of Hd3a and RFT1 (another FT ortholog; Komiy a et al., 2008) but had no effect on the expression profile of Hd1 (Fig. 2, I–K). To further confirm this genetic interaction, we analyzed the expression of Hd1, Hd3a, and RFT1 in an Hd1 nonfunctional background [NIL(Hd1) and NIL(Hd1,6)]. Hd3a and RFT1 were not dramatically repressed by Hd6 in the Hd1 nonfunctional background under LD conditions, although there seemed to be some Hd3a repression by Hd6 in the hd1 background (Supplemental Fig. S4). These results suggested that Hd6 modulates Hd1 repression activity mainly by posttranslational modification.
Hd6 Has Protein Kinase Activity But Does Not Phosphorylate Hd1 in Vitro

To confirm the protein kinase activity of Hd6, we measured the CK2 activity of the Hd6 recombinant protein (rHd6; Sugano et al., 1998). We expressed Hd6 as a glutathione S-transferase (GST) fusion protein and isolated it from GST by thrombin treatment of the purified fusion protein bound to glutathione agarose beads (Supplemental Fig. S5A). We then measured the CK2 activity of the isolated rHd6. Purified rHd6 had nearly the same CK2 activity as a control human CK2α (Supplemental Fig. S5B).
To investigate Hd1 phosphorylation, GST fusion Hd1 protein was purified as above and its potential as an Hd6 substrate was characterized in vitro. Hd1 was not phosphorylated by rHd6 in vitro (Supplemental Fig. S6). The observation that flowering regulation by H6O required Hd1 and that Hd1 was not a direct Hd6 target suggests that Hd6 protein phosphorylates an unknown flowering repressor target that works together with Hd1.

Overexpression of a Dominant-Negative Form of Hd6 Causes Early Flowering under LD Conditions

By a genome-wide homology search in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/), we found four genes with very high levels of identity to CK2α subunit genes in the rice genome (Supplemental Fig. S7A). Expression analysis revealed that, of the four CK2α genes, functional H6O (OsCKA2-1) and OsCKA2-2 were the two main ones expressed in various tissues of rice (Supplemental Fig. S7, B and C). Therefore, with deficiency of the H6O gene alone it was not easy to draw conclusions about the overall role of phosphorylation by CK2 in rice biology. Therefore, we used H6OTik, the Timekeeper (Tik) mutant form of Hd6, a dominant-negative CK2α allele that was originally identified in Drosophila (Akten et al., 2003; Moreno-Romero et al., 2008), to elucidate the role of CK2 in rice (Fig. 3A). We measured the CK2 activity of recombinant H6OTik and confirmed that the mutant protein had greatly reduced CK2 activity (Supplemental Fig. S5B). Next, we used the cauliflower mosaic virus (CaMV) 35S promoter to generate transgenic rice overexpressing H6OTik. With these H6OTik lines, we were able to reduce the total in vitro CK2 phosphorylation activity in cell extracts to about half of that observed with the nonfunctional H6O allele (Fig. 3B).

Overexpression of the dominant-negative form (Allada and Meissner, 2005; Moreno-Romero et al., 2008; Smith et al., 2008) of H6O resulted in early flowering equivalent to that observed in plants with Hd1-defective alleles under LD conditions (Fig. 3, C and D). Some of the H6OTik lines failed to thrive in the T1 generation, especially under SD conditions, suggesting that CK2 has pleiotropic functions in rice, as recently found in Arabidopsis by the use of transgenic lines carrying a dominant-negative mutation in a CK2α allele and an antisense construct (Lee et al., 2009; Smith et al., 2008). Pleiotropic effects such as retarded growth were indeed observed in H6OTik lines (Fig. 3, E–I). We also observed slightly later flowering phenotypes in H6OTik lines than in hd1-deficient NIL plants under SD conditions (Fig. 3C), suggesting that there is extra floral control by CK2 that is independent of Hd1. In addition, Hd1 mRNA expression was reduced under LD conditions in H6OTik lines (Fig. 3, J–M), suggesting the involvement of CK2 in transcriptional control of Hd1. However, the occurrence of pleiotropic effects in the H6OTik lines (Fig. 3, E–I) does not support this possibility. To fully understand the mechanisms underlying the functions of CK2, further investigations of the biological roles of CK2, other than its role in photoperiodic flowering in rice, are required.

Overexpression of H6OTik Does Not Affect the Circadian Clock

To determine the effects of CK2 loss of function on molecular clock rhythms in rice, we next tested whether overexpression of a dominant-negative form of H6O altered the rhythmic expression of OsPRR1. The expression pattern of OsPRR1 in rice cells was monitored by LUC activity (Supplemental Fig. S8). H6OTik did not affect OsPRR1:LUC expression under light/dark and constant dark conditions (Fig. 4). By comparison, CK2 activity was essential for Hd1 activity in both promotion of flowering under SD conditions and repression of flowering under LD conditions (Fig. 3, C and D). These results indicated that the role of CK2 in photoperiodic flowering in rice is distinct from the role of CK2 reported in Arabidopsis (Sugano et al., 1999; Daniel et al., 2004) and that CK2 does not contribute markedly to regulation of the circadian clock in rice (Figs. 2–4).

CK2 Phosphorylation Sites in OsLHY Are Evolutionarily Conserved, Except One Found in CCA1

In Arabidopsis, CCA1 clock protein phosphorylation by CK2 is necessary for proper action of the circadian clock (Daniel et al., 2004). We started to investigate the mechanism underlying our finding by examining the phosphorylation sites in OsLHY proteins. First, GST-OsLHY protein was phosphorylated in vitro by rHd6 (Fig. 5). Deletion and amino acid exchange analysis revealed that OsLHY contained five phosphorylated Ser sites in two OsLHY domains, C5 and C3 (Okada et al., 2009; Figs. 5 and 6; Supplemental Fig. S9). We compared the corresponding positions of Ser sites between CCA1 and OsLHY and found that Ser-484 of CCA1 corresponded to Glu-600 of OsLHY, although five Ser sites (Ser-5, Ser-6, Ser-550, Ser-551, and Ser-552) in OsLHY were conserved as the corresponding sites in CCA1 (Fig. 6). We further compared the public database amino acid sequences of 22 CCA1 orthologs from 16 genera and found several motifs conserved in angiosperms (Fig. 6; Supplemental Figs. S9 and S10; Supplemental Tables S1 and S2). Structurally, the LHY/CCA1-like genes are typified by the presence of nine highly conserved domains (C1–C9) in higher plants. Four domains (C1–C4) are conserved in moss (Okada et al., 2009). All the Ser sites in CCA1 were located in conserved domains (C5, C3, and C4). The Ser site in the C4 domain was changed to Glu in rice OsLHY (Fig. 6). Compared with other domains related to basic helix-loop-helix, the C4 domain was found only in CCA1, LHY, and OsLHY among all the annotations in the Arabidopsis and rice genomes, suggesting that the C4 domain is evolution-
Figure 3. Phenotypes of rice overexpressing a dominant-negative CK2α allele. A, The sequence on top shows the Tik mutation in a dominant-negative form of CK2α, with mutations of M158K and E162D sites in H6, referred from the Tik allele of Drosophila melanogaster (in red). B, CK2 activity in plant extracts. Data are means of three experiments for each line using distinct tillers after flowering. C and D, Flowering times in Nipponbare, NIL(\(Hd1\)) NIL\((Hd6)\), NIL\((Hd1,6)\), H6ox, and H6Tikox were examined. T1 plants were grown under SD (10 h of light/14 h of dark) and LD (14.5 h of light/9.5 h of dark) conditions. Histograms for days to flowering are shown. Rice plants of NIL\((Hd6)\) and H6ox did not flower within 100 d after sowing under LD conditions. Black bars indicate ranges of flowering time for the tested lines (i.e. that all tested plants flowered within these ranges). E to I, Nipponbare, NIL\((Hd1)\), H6Tikox, and H6ox plants under LD conditions 70 d after sowing. All tillers were removed. Most of the surviving H6Tikox T1 plants exhibited normal growth (E and F), but a few plants showed abnormal phenotypes (e.g., arrowhead in E). G, H6Tikox plants (red arrowhead) 30 d after sowing. H and I, Retarded panicles in H6Tikox plants. J to M, Gene expression in H6Tik lines.
arily associated with the plant circadian clock (Fig. 7; Supplemental Fig. S9).

Biological Roles of CK2 Phosphorylation Sites in OsLHY

As described above, when the C4 domain was subjected to a homology search, we found that Glu (in Glu-600) was a major amino acid in the C4 domain and that Ser (in Ser-484) in CCA1 was an exception (Figs. 6 and 7). This conserved C4 domain fragment in OsLHY was not phosphorylated but that in CCA1 was, indicating that there was no Ser site for compensation for Ser-484 of CCA1 in the C4 domain of OsLHY (Fig. 5, A and C). A yeast two-hybrid (Y2H) assay further revealed that only the Ser-484 site, not the other five Ser sites, was critical for CCA1 homodimer formation (Fig. 8A). In contrast, OsLHY did not form a homodimer in the Y2H assay, suggesting that OsLHY and CCA1 had distinctly different biochemical properties. Although overexpression of tagged OsLHY was able to repress OsPRR1:Luc rhythmic expression in rice calli, that of tagged mOsLHY(A) (an E600A mutant of OsLHY) was not able to repress it (Fig. 8, B and C). These results suggested that OsLHY Glu-600 is essential for circadian clock function. In addition, to try to evaluate OsLHY stability in rice, we made transgenic rice calli overexpressing an OsLHY-LUC fusion protein driven by a maize (Zea mays) ubiquitin promoter (Fig. 9). The LUC activity of the OsLHY-LUC protein exhibited diurnal rhythms under light/dark conditions but no rhythms (and clear damping) under constant dark conditions. This strongly suggested that OsLHY protein becomes stable under light conditions and unstable under dark conditions. In contrast, OsLHY-LUC(5A), with five Ser-to-Ala mutations at the five conserved Ser sites in OsLHY, completely lost the light stabilization of OsLHY-LUC, suggesting that Glu-600 was not involved in the control of OsLHY stabilization by light. It is also possible that this

Figure 4. Hd6Tik did not markedly affect circadian rhythmic expression of OsPRR1::Luc. Independent transformed 35S:Hd6Tik calli carrying the same OsPRR1::Luc reporter gene were measured for OsPRR1::Luc in constant darkness (DD). Data from the original OsPRR::Luc line (control) and transformed 35S:Hd6 calli are presented together. Data for the period 48 to 60 h were missed accidentally.

Figure 5. CK2(rHd6) phosphorylated two regions in OsLHY. A, Schematic images of OsLHY and OsLHY fragments and OsLHY with mutations at all five possible phosphorylation sites from Ser to Ala, termed OsLHY(5A). Arrowheads indicate putative CK2 consensus sites (S/TXXD/E) found in OsLHY. Red arrowheads indicate five Ser residues at the phosphorylation sites corresponding to those in CCA1. Deleted OsLHY fragments are designated by the corresponding amino acid residue numbers in full-length OsLHY. The CK2 target assay results are summarized at the right. B, SDS-PAGE and autoradiograph of SDS-PAGE analyses of GST-CCA1, GST-OsLHY, and GST-mOsLHY(5A) after in vitro CK2 (rHd6) phosphorylation assays. C, SDS-PAGE and autoradiographs of SDS-PAGE analyses of the C-terminal segments of OsLHY fused to GST after in vitro CK2 (rHd6) phosphorylation assays. A fragment containing the C4 domain region (568–719) of OsLHY was not phosphorylated, whereas the corresponding CCA1 fragment (449–608) was phosphorylated.
rhythm of LUC activity came from translational regulation and protein modification.

DISCUSSION

Using rice NILs and lines overexpressing Hd6, we demonstrated here that flowering-time control by the previously identified quantitative trait locus, Hd6, in rice requires a functional Hd1 gene (an Arabidopsis CONSTANS ortholog) but does not occur through circadian clock action (Figs. 1 and 2; Supplemental Figs. S2 and S4). Because the Hd6 gene is a member of the CK2α subfamily and is one of two major expressed CK2α subunits (Supplemental Fig. S7), transgenic lines overexpressing a dominant-negative CK2α were further examined to evaluate the role of CK2 in the circadian clock in the photoperiodic control of flowering in rice. Because CK2 activity was not completely absent in these dominant-negative lines (Fig. 3), we were able to conclude that circadian clock activity was disconnected from the control of flowering time by Hd6 but not from all CK2 activity. However, we confirmed that the circadian clock in rice was less affected by a reduction in CK2 activity than was flowering-time control (Figs. 3 and 4). We did not identify the target protein that could be phosphorylated by Hd6 CK2α to control flowering time. Our genetic analysis, however, strongly suggested that any protein that could work together with Hd1 might be a target. Recently, the Ghd7 gene was reported to be a strong repressor of rice flowering (Xue et al., 2008). We found that Hd6 did not phosphorylate either Hd1 or Ghd7 protein in vitro (Supplemental Fig. S6; data not shown). In addition, our preliminary data revealed that some protein associated with Hd1 in vitro could be an Hd6 target for the control of Hd1 repressor activity (data not shown). We are now attempting to identify this Hd6 target.

Although there is no genetic evidence of a role for OsLHY in the circadian clock in rice, we demonstrated here that overexpression of tagged OsLHY repressed the rhythmic expression of OsPRR1:LUC in rice cells (Fig. 8C). This result strongly suggested that OsLHY is a core component of the circadian clock in rice and was consistent with the concept of OsLHY as a sole ortholog of Arabidopsis CCA1/LHY in the rice genome. Notably, we tried a simple way of making transgenic rice plants overexpressing OsLHY cDNA (without the tag) driven by both CaMV 35S and a maize ubiquitin promoter, but we were not able to obtain any rice plants overexpressing OsLHY mRNA (data not shown). In addition, we succeeded in overexpressing the OsLHY-LUC gene in rice cells (Fig. 9), but no associated phenotypes were observed. We obtained circadian clock-related phenotypes associated with the repression of OsPRR1:LUC rhythmic expression, but only when we overexpressed the tagged OsLHY. Unlike with LHY/CCA1, the simple overexpression of which disturbs the circadian clock in Arabidopsis (Schaffer et al., 1998; Wang and Tobin, 1998), in the case of OsLHY its function may be too critical in rice to obtain a simple overexpressor.

We next revealed that OsLHY could not form a homodimer in yeast, whereas CCA1 could do so through the C4 domain (Ser-484 site), one of the previously identified CK2 phosphorylation sites (Daniel et al., 2004; Fig. 8A; data not shown). This finding is consistent with that of a previous report in Arabidopsis (Daniel et al., 2004). By comparison, the corresponding site (Glu-600) in OsLHY in rice was critical for repression of the rhythmic expression of OsPRR1.

In Arabidopsis, the findings that lines overexpressing CKB3 and CKB4 have circadian clocks with short free-running periods and show early flowering under SD conditions indicate that CK2 can control the circadian clock and flowering time in this species and that this control is mediated by CCA1 phosphorylation (Sugano et al., 1999; Daniel et al., 2004). CCA1 and LHY function as TOC1/PRR1 repressors (Mizuno and Nakamichi, 2005). These actions form a negative feedback loop in the circadian clock of Arabidopsis (Alabadi et al., 2001; Mizuno and Nakamichi, 2005). As expected, OsLHY expression is likely to be regulated by CK2 phosphorylation.
CCA1 phosphorylation is necessary for CCA1 homodimerization and normal clock function, OsLHY phosphorylation is likely to be important for rice circadian clock function. In fact, OsLHY was phosphorylated by Hd6, and the phosphorylation sites were conserved, with one exception (Figs. 6 and 7; Supplemental Table S3). However, NIL(Hd6) lines, Hd6-overexpressing lines, and dominant-negative lines had little effect on the rice circadian clock (Figs. 2 and 4). Only one unconserved site, OsLHY Glu-600, corresponding to CCA1 Ser-484, was present in a highly conserved motif, termed the C4 domain in land plants (Fig. 7). CCA1 Ser-484 is necessary for homodimerization (Fig. 8A). In addition, mOsLHY E600A overexpression did not repress OsPRR1:LuC in rice calli, whereas OsLHY overexpression did (Fig. 8, B and C; data not shown). These results indicate that this Glu site in the C4 domain was critical for OsLHY function related to the circadian clock. They also strongly suggest that the change from Ser to Glu at the Glu-600 site in OsLHY is a critical cause of the disconnection of Hd6 from rice circadian clock regulation. During plant evolution, CCA1 may have acquired the capacity for Ser-484 phosphorylation, which may then have led to dimer formation for fine regulation of the circadian clock in Arabidopsis. The inability of OsLHY to form homodimers (Fig. 8A) suggests that OsLHY has a role in

Figure 7. Multiple alignment of C4 domain sequences in plants. This plant-specific motif is found only in LHY/CCA1 and OsLHY in the Arabidopsis and rice genomes, respectively. Likewise, this C4 domain is found only in predicted LHY-like gene(s) regions in the Sorghum, Populus, Vitis vinifera, and Physcomitrella genomes, respectively (Supplemental Fig. S9). Glu (E) is the amino acid site found in this domain, and one that corresponds to a CK2 phosphorylation site in CCA1. This amino acid is found in all C4 domain-like sequences in various plant species EST databases (Supplemental Table S4), with the exception of three species of eudicots, two of magnolids, and one of monocots (arrow).
regulation of the circadian clock system in rice and that this role is distinct from that of CCA1 found in Arabidopsis.

Interestingly, of the other five Ser sites phosphorylated in vitro by rHd6, those corresponding to CCA1 sites might be involved in the light-dependent protein stability of an OsLHY-LUC fusion protein (Fig. 9), although the molecular nature of the rhythms of OsLHY-LUC has not been confirmed. This result for OsLHY-LUC suggests that OsLHY activity under daily light/dark cycles is controlled by phosphorylation by CK2 (or other kinases) and that control of this nature, through the sites of phosphorylation of OsLHY, is clearly distinct from the control provided by the Glu-600 and Ser-484 sites in OsLHY and CCA1. A, Y2H assays. Mutations in CCA1 altered the interactions with CCA1 in yeast. The Ser-484 site in CCA1 was critical for homodimer formation of CCA1 in yeast. OsLHY did not interact with OsLHY itself in yeast. All experiments were done in accordance with the manufacturer's instructions for the Matchmaker kit (BD Clontech). Average values of β-galactosidase activities from five individual colonies from a representative experiment are shown ± SE at right. B, Schematic view of tested constructs with site-specific mutations. The arrow indicates the position of Glu/Ser in the C4 domain. The identified CK2 target sites are shown as asterisks for CCA1 and OsLHY. C, Bioluminescence analysis of OsPRR1:Luc expression in rice calli transformed with UBQ:OsLHY(A)-3FLAG or UBQ:mOsLHY(E600A)-3FLAG in light/dark (LD) followed by constant darkness (DD) cycles. About 10 independent transformed T0 cell lines carrying the same OsPRR1:Luc reporter gene were measured. Average counts are shown.

Figure 8. Biochemical and biological roles of the Glu-600 and Ser-484 sites in OsLHY and CCA1. A, Y2H assays. Mutations in CCA1 altered the interactions with CCA1 in yeast. The Ser-484 site in CCA1 was critical for homodimer formation of CCA1 in yeast. OsLHY did not interact with OsLHY itself in yeast. All experiments were done in accordance with the manufacturer’s instructions for the Matchmaker kit (BD Clontech). Average values of β-galactosidase activities from five individual colonies from a representative experiment are shown ± SE at right. B, Schematic view of tested constructs with site-specific mutations. The arrow indicates the position of Glu/Ser in the C4 domain. The identified CK2 target sites are shown as asterisks for CCA1 and OsLHY. C, Bioluminescence analysis of OsPRR1:Luc expression in rice calli transformed with UBQ:OsLHY(A)-3FLAG or UBQ:mOsLHY(E600A)-3FLAG in light/dark (LD) followed by constant darkness (DD) cycles. About 10 independent transformed T0 cell lines carrying the same OsPRR1:Luc reporter gene were measured. Average counts are shown.
600 site of OsLHY for circadian clock oscillation in rice, which confers rhythmic gene expression under constant light or dark conditions. The molecular nature of light-regulated translation of LHY protein has also been reported in Arabidopsis (Kim et al., 2003). Therefore, the regulation of protein stability by light in LHY and OsLHY is likely to be conserved among plant species through phosphorylation of the five conserved amino acids by CK2. The control of light-dependent protein stability (Kim et al., 2003) by the phosphorylation Ser sites in OsLHY implies that the robustness of diurnal rhythmic expression of OsLHY is an evolutionarily conserved function. The contribution of CK2 to circadian clock regulation under constant light or dark conditions might be a recently developed function of CK2 observed in Arabidopsis. The finding that Glu was a major amino acid at Glu-600 in OsLHY (corresponding to Ser-484 in CCA1) strongly supports this idea. These findings might shed light on the molecular mechanisms underlying the development of circadian clock systems and photoperiodic flowering during plant evolution, since Arabidopsis and rice are believed to have diverged about 200 million years ago (Fig. 10).

Figure 9. Light-dependent OsLHY protein stability in transgenic rice calli. A, Structure of OsLHY-LUC used for this experiment. B, Four distinct transgenic cell lines carrying UBQ: LUC, UBQ:OsLHY-LUC, UBQ:OsLHY(A)-LUC, and UBQ:OsLHY(5A)-LUC were grown under 12-h-light/12-h-dark conditions (12:12LD), and their LUC bioluminescence activity was monitored for 1 week under 12:12LD conditions and 3 d under constant darkness (DD) conditions. Light dependency of LUC stability was observed in UBQ:OsLHY-LUC lines. The quintuple mutations in the phosphorylation sites of OsLHY in the mOsLHY(5A) transgenic line may have resulted in loss of the light dependency of fusion protein stability in rice cells. By comparison, the Ala mutation at the Glu site in mOsLHY(A) did not greatly affect LUC activity. Similar bioluminescence profiles were reproducibly obtained. A representative result is shown.

Figure 10. Model of CK2(Hd6) regulation of the circadian clock and photoperiodic control of flowering in Arabidopsis and rice. In Arabidopsis, CK2 may control the circadian clock through CCA1 dimer formation. In rice, it is likely that CK2 does not control the circadian clock, since OsLHY (an ortholog of CCA1) cannot form homodimers and the amino acid site corresponding to the CK2 target site of CCA1 has been changed to Glu. It is still possible that phosphorylation of OsLHY by CK2 controls protein stability. In addition, CK2 may control Hd1 activity through the phosphorylation of an unknown interacting factor to control flowering time in rice.
MATERIALS AND METHODS

Plant Materials and Growth Conditions for Measuring Flowering Time

One rice (Oryza sativa) cultivar, three NILs (Supplemental Fig. S1) that contained an Hdl or Hdl chromosomal region of the Kasalath genome, and two overexpression lines were used. The DNA markers for selection of the Kasalath fragments for the Hdl and Hdl regions were Arg-2171 to Arg-2654 and Arg-2311 to Cys-217, respectively. The constructs for the overexpression lines were built in the following way. Nipponbare (genotype Hdl/Hdl), NIL(Hdl) (genotype Hdl/Hdl), NIL(Hdl)1) (genotype Hdl/Hdl), and Hailory transgenic [genetic background: Nipponbare and NIL(Hdl)] plants (Supplemental Fig. S1) were grown under SD (10 h of light/14 h of dark) or LD (14 h of light/10 h of dark or 14.5 h of light/9.5 h of dark) conditions. All tillers after the fourth were removed to save space under LD conditions. A metal halide lamp was used as the light source in the growth chambers. Photosynthetic photon flux density ranged from 450 to 500 μmol m⁻² s⁻¹.

Establishment of Rice Cell Lines for Bioluminescence Analysis

Rice cells were transformed with the OsPRR1::LUC construct using G418 as a selection marker, and the cell lines obtained were selected on the basis of stable rhythmic expression of OsPRR1::LUC in constant darkness. The selected OsPRR1::LUC cell line was then further transformed with plasmids [35S: OsLHY-3FLAG, 35S:OsLHY(A)-3FLAG, etc.]. The effects of OsPRR1::LUC expression were examined in about 10 independent cell lines.

Bioluminescence Assays of Circadian Rhythm

Transgenic lines containing cab:LUC together with Hdl were selected in the F5-471 (the F5 progeny from a cross between a japonica rice with an hdl allele, Norin8, carrying cab:LUC, Suguymama et al., 2001) and another japonica line with an Hdl allele, Kinnazae. A few seedlings were grown with 3 mL of Murashige and Skoog medium in a 35×10-mm petri dish (Becton-Dickinson) and entrained for 5 d in 12-h-light/12-h-dark cycles at 30°C. The light source was a mixture of red and blue light-emitting diode lamps (Sanyo) with a photon flux density of 200 μmol m⁻² s⁻¹. The bioluminescence in transformed cell lines (callus) containing OsPRR1::LUC was measured in a 90×20-mm petri dish (Bio-Bik). An Aequorosmos photon-counting system (Hamamatsu Photonic Systems) was used for all bioluminescence imaging experiments. Periods and relative amplitude errors were estimated from data starting from 24 h after the last dark-to-light transition using fast Fourier transform-nonlinear least squares software, as described by Plaut et al. (1997).

Constructs

All primer sequence information is given in Supplemental Table S1. Plasmids for overexpressing the target gene product were constructed using either a pBl vector containing the CaMV 35S promoter or a related pSk2 vector (Izawa et al., 2002) containing a maize (Zea mays) ubiquitin promoter. Constructs for overexpressing the Hdl cDNA fragment and the mutated Hdl cDNA fragment were generated by means of the megaprimer method using a full-length Hdl cDNA clone as template. The primer sets used were Hdl-1F and Hdl-R for Hdl and Hdl-1F and Hdl-Tik-R2, Hdl-Tik-F2 and Hdl-R for Hdl. Rice line Nipponbare was used for transformation in making these overexpressers. The 35S-strepIII-3xFLAG-Hdl1 construct was generated from PCR fragments using designed oligonucleotides. For the 3xFLAG tag, the following primers were used: FlagNF1, FlagNF2, FlagN1R1, FlagN2R1, FlagN2R2, FlagN3R1, and FlagN3R2 (Supplemental Table S1). For the strepIII tag, the following oligonucleotide linkers were used: XhoI-strepIII-BamHI-F and XhoI-strepIII-BamHI-R. Constructs with mutated OsLHY and CCA1 [OsLHY(A), CCA1(484A), CCA1(484E), CCA1(5A), and CCA1(6A)] were generated by the megaprimer method (Sarkar and Sommer, 1990) and amplified using full-length OsLHY and CCA1 clones as templates. The primer sets used were as follows: OsLHY-5,6-S-A-A-F and OsLHY-5,6-R and OsLHY-5,6-F and OsLHY-600-E-A, R, OsLHY-600-A-F and OsLHY-5,6-R and OsLHY-600-E-A, R, OsLHY-600-A-F and OsLHY-5,6-R and OsLHY-600-E-A, R, and OsLHY-5,6-A-A-F and CCA1-S-A-A-R for CCA1(484A), CCA1-S-A-E-F and CCA1-S-E-R for CCA1(484E), CCA1-56-7-S-A-F for CCA1(56,57A), and CCA1-S-A-A-F and CCA1-S-A-A-R for CCA1(5A) and CCA1(6A). All constructs containing OsLHY or CCA1, as well as the final mutated binary constructs, were verified by sequence analysis of inserts. For Hdl phosphorilation tests using Escherichia coli-produced proteins, pGEX-OsLHY, mOsLHYs, and OsLHYs, the deletion lines, and related CCA1 constructs (GST fusion proteins) were generated as above. Initially, full-length OsLHY and CCA1 cDNA subclones were digested with Sall and NotI and ligated into pGEX-4T vector (GE Healthcare Life Sciences). The OsPRR1:LUC construct was generated by PCR with primers KpnI-OsPRR1pro-F and HindIII-OsPRR1ex2-R. These fragments were fused to the modified firefly LUC+ gene on pSP-luc (Promega) at the KpnI and HindIII sites. The OsPRR1:LUC constructs were cloned onto the KpnI and SacI sites of an Agrobacterium tumefaciens binary vector, pBINPLUS. Nipponbare was transformed by a typical Agrobacterium-mediated method. We isolated several independent transgenic cell lines with OsPRR1:LUC. To generate the UBG-OsLHY-3FLAG and UBG-mOsLHY(A)-3FLAG constructs, non-stop-codon OsLHY was amplified by PCR with the primers OsLHY-SalI-F and OsLHY-strepXhoI-R. These isolated fragments were fused to TOPO vector (Invitrogen), and Sall/NotI fragments were fused to pBII21 vector. To generate 3xFLAG fusion OsLHY, 3xFLAG oligonucleotide linker was ligated into the C terminus of OsLHY (Xhol/NotI) using Xhol-3FLAG-F and Xhol-3FLAG-R and NotI-3FLAG-F and NotI-3FLAG-R. The OsLHY-3FLAG-nos and mOsLHY(A)-3FLAG-nos constructs were cloned onto the SalI and EcoRI sites of an Agrobacterium binary vector, pLP2-UBQ. The calli of one line of OsPRR1:LUC were selected and transformed by the same Agrobacterium-mediated method with a different selectable antibiotic marker. We isolated several independent transgenic cell lines.

Real-Time Quantitative PCR

Total RNA was prepared by a conventional SDS-phenol method. The reverse transcription (RT) reaction was performed with SuperScript II reverse transcriptase (Invitrogen), oligo(dT)₁₂₋₁₈ (Invitrogen), and 5 μg of total RNA in accordance with the manufacturer’s instructions. Real-time quantitative RT-PCR analysis was performed as described previously. Briefly, quantitative RT-PCR was performed by the Taq-Man PCR method using an ABI PRISM 7900 Sequence Detection System in accordance with the manufacturer’s instructions. Primer and probe sequence information for the quantitative RT-PCR is listed in Supplemental Table S4.

CK2 Activity Assay

One hundred milligrams of frozen seedlings was ground and extracted with 100 μL of extraction buffer (50 mM Tris-HCl pH 7.6, 15 mM MgCl₂, 0.1 mM KCl, 0.25 mM Suc, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture [Sigma], phosphatase inhibitor mixture [Sigma], and 14 mM 2-mercaptoethanol). After centrifugation of the sample at 15,000 rpm for 10 min, the supernatant was sampled and the protein concentration was measured. CK2 activity in these extracts was measured with a CK2 kinase assay kit (Upstate) in accordance with the manufacturer’s instructions.

Recombinant Protein Purification

GST-Hdl, GST-OsLHY, and GST-mOsLHY series and GST-CCA1 fusion proteins were overexpressed in E. coli (strain BL21) and purified. To remove the GST affinity tail from the GST-Hdl fusion protein, protease cleavage by biotinylated thrombin protease combined with Glutathione-Sepharose 4B (Novagen) was used in accordance with the manufacturer’s instructions. After the removal of thrombin by the addition of streptavidin agarose, the recombinant Hdl protein was stored at 20°C until required for the in vitro assay to detect Hdl target proteins.

Y2H Assay

The protein-coding sequences of OsLHY, mOsLHYs, CCA1, and mCCA1s were cloned into the plasmids provided in the Matchmaker GAL4 Two-Hybrid System 3 kit (BD Clontech). All assays were performed in accordance with the manufacturer’s instructions. Expression of all proteins was confirmed by immunoblot analysis.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Graphical genotypes of NILs.
Supplemental Figure S2. Functional Hd1 gene is necessary for the late flowering observed in Hd6-overexpressing lines under LD conditions.

Supplemental Figure S3. Hd6ox does not affect the rhythms of clock-controlled gene expression in continuous light (LL).

Supplemental Figure S4. Hd6 does not repress Hela and RFTI critically in an hd1 nonfunctional background.

Supplemental Figure S5. Activity of purified recombinant Hd6 and Hd6\(^{\text{Hk}}\) proteins expressed in E. coli.

Supplemental Figure S6. CK2(Hd6) phosphorylation assay for Hd1.

Supplemental Figure S7. CK2α genes in rice.

Supplemental Figure S8. Circadian clock monitoring by using OsPRR1: LUC in rice cells.

Supplemental Figure S9. Multiple alignment of LHY, CCA1, OsLHY, and LHY-like proteins.

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

Supplemental Table S1. C1 to C9 domains and conserved CCA1 phosphorylation sites from LHY/CCA1-like proteins in angiosperms.

Supplemental Table S2. C4 domain-like sequences in database.

Supplemental Table S3. Primers used for constructs in this work.

Supplemental Table S4. Primers and Taq-Man probes for quantitative RT-PCR.

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Supplemental Table S1. Conserved CCA1 phos-
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