Casein Kinases I and 2α Phosphorylate Oryza Sativa Pseudo-Response Regulator 37 (OsPRR37) in Photoperiodic Flowering in Rice

Choon-Tak Kwon¹, Bon-Hyuk Koo¹, Dami Kim¹, Soo-Cheul Yoo², and Nam-Chon Paek¹,*

Flowering time (or heading date) is controlled by intrinsic genetic programs in response to environmental cues, such as photoperiod and temperature. Rice, a facultative short-day (SD) plant, flowers early in SD and late in long-day (LD) conditions. Casein kinases (CKs) generally act as positive regulators in many signaling pathways in plants. In rice, Heading date 6 (Hd6) and Hd16 encode CK2α and CKI, respectively, and mainly function to delay flowering time. Additionally, the major LD-dependent floral repressors Hd2/ Oryza sativa Pseudo-Response Regulator 37 (OsPRR37; hereafter PRR37) and Ghd7 also confer strong photoperiod sensitivity. In floral induction, Hd16 acts upstream of Ghd7 and CKI interacts with and phosphorylates Ghd7. In addition, Hd6 and Hd16 also act upstream of Hd2. However, whether CKI and CK2α directly regulate the function of PRR37 remains unclear. Here, we use in vitro pull-down and in vivo bimolecular fluorescence complementation assays to show that CKI and CK2α interact with and phosphorylate PRR37. We further use in vitro kinase assays to show that CKI and CK2α phosphorylate different regions of PRR37. Our results indicate that direct posttranslational modification of PRR37 mediates the genetic interactions between these two protein kinases and PRR37. The significance of CK-mediated phosphorylation for PRR37 and Ghd7 function is discussed.

INTRODUCTION

In plants, complex interactions between endogenous circadian clock components and environmental factors trigger flowering; these environmental factors include seasonal changes in day length (photoperiod) and temperature. To date, research in model systems and crops has identified many regulatory genes controlling flowering time (also known as heading date), for example in the dicot Arabidopsis thaliana and the monocot rice (Oryza sativa).

Cultivated rice is a facultative short-day (SD) plant that flowers early in SD (<10-h light/day) and late in long day (LD; >14-h light/day) conditions (Izawa, 2007; Tsuji et al., 2008). Modern rice cultivation spans geographical latitudes from 53°N to 40°S and photoperiod sensitivity affects adaptation for growth at these different latitudes, significantly affecting grain yield (Izawa, 2007). For example, japonica rice in the northern-limit regions (>40°N) generally flowers extremely early, which results in proper grain production under natural LD conditions in the short summer period (Fujino and Sekiguchi, 2005; Wei et al., 2008). In Arabidopsis, four major pathways regulate flowering time: photoperiod, gibberellin, vernalization, and autonomous pathways. In LD conditions, the GIGANTEA (GI)-CONSTANS (CO)-FLOWERING LOCUS T (FT) module is the major photo-period pathway for floral induction (Huq et al., 2000; Kardailsky et al., 1999; Kobayashi et al., 1999; Park et al., 1999). The flowering FT moves from the leaves to the shoot apex and binds to the bZIP transcription factor FD to activate the expression of floral meristem-identity genes (Abe et al., 2005; Corbesier et al., 2007). In rice, OsGI-Heading date 1 (Hd1)-Heading date 3a (Hd3a) form a conserved pathway that functions in floral induction, mainly in SD (Hayama et al., 2003; Yano et al., 2000). In addition, the OsMADS50-Early heading date 1 (Ehd1)-Rice flowering locus T1 (RFT1) pathway also functions in floral induction, mainly in LD (Doi et al., 2004; Komiya et al., 2009). The rice floigens Hd3a and RFT1 activate the expression of the floral meristem-identity genes OsMADS14 and OsMADS15 (Komiya et al., 2008). In the two principal pathways of floral induction, Days to heading 8 (DTH8)/Grain number, plant height and heading date 8 (Ghd8)/Hd5 suppresses Ehd1 expression to inhibit flowering in LD (Fujino et al., 2013; Wei et al., 2010; Yan et al., 2011). Ghd7 functions as one of the major LD-dependent floral repressors, downregulating Ehd1 expression (Xue et al., 2008). By contrast, Rice Indeterminate 1 (OsID1)/ Ehd2/RID1 (Matsubara et al., 2009b; Park et al., 2008; Wu et al., 2008) and Ehd3 (Matsubara et al., 2011) function as major positive regulators, upregulating Ehd1 expression to promote flowering in LD. Recent work has identified several genes up-stream of Hd1 and Ehd1; for example, Hd17/OsELF3, the rice ortholog of Arabidopsis ELF3, functions as a floral inducer by downregulating Ghd7 expression (Matsubara et al., 2012; Saito...
et al., 2012; Yang et al., 2013; Zhao et al., 2012). Ehd4 acts upstream of Ehd1 and positively regulates the expression of Hd3a and RFT1 by increasing Ehd1 expression (Gao et al., 2013). By contrast, DTH2, which encodes a CO-like protein, promotes flowering by upregulating the expression of Hd3a and RFT1 independent of Hd1 and Ehd1 (Wu et al., 2013).

In Arabidopsis, direct protein-protein interactions play crucial roles in the control of flowering time. For example, initiation of floral development in the shoot apex requires the interaction between FT and FD (Abe et al., 2005). In LD, FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) interacts with GI and the FKF1-GI complex activates CO expression in the late afternoon (Sawa et al., 2007). FKF1 also interacts with CO through its LOV domain and stabilizes CO binding to the FT promoter (Song et al., 2012). During the night, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) interacts with and destabilizes CO. In SD, ELF3 interacts with COP1 and GI; this allows COP1 to ubiquitinate ELF3 and GI for COP1-ELF3-GI degradation via the 26S proteasome (Yu et al., 2008).

Furthermore, ELF3 also forms a complex with LUX ARRHYTHMO (LUX) and ELF4; the ELF4-ELF5-LUX complex forms transiently at dusk to downregulate the expression of PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF5 for diurnal hypocotyl growth (Herrero et al., 2012; Nusinow et al., 2011). In contrast to Arabidopsis, most of the protein-protein interactions are revealed during flowering-time regulators in rice remain to be examined. One recent example showed that Hd3a binds to 14-3-3; their complex moves to the nucleus and interacts with OsFD1 to induce floral meristem-identity genes (Taoka et al., 2011).

The highly conserved serine/threonine-specific casein kinases (CKs) control various signal transduction processes in eukaryotes (Knippschild et al., 2005; Mulekar and Huq, 2014). In plants, CKI and CK2 affect the regulation of flowering time and circadian rhythm. In Arabidopsis, CK1.3 and CK1.4 have important roles in the regulation of blue-light signaling and circadian rhythm by decreasing the stability of cryptochrome 2 through phosphorylation (Tan et al., 2013). Moreover, the constitutive circadian clock component CK2 positively regulates the bluelight signaling and circadian rhythm. In Arabidopsis, the major LD-dependent floral repressors, together with Ghd7 (Xue et al., 2008), both of which play an important role in photoperiod sensitivity in rice (Koo et al., 2013; Murakami et al., 2005; Yan et al., 2013).

Here, we show the direct interactions of Hd6/CK2α and Hd16/CKI with Hd2/PRR37 in vitro and in vivo. We also show that these two CKs phosphorylate different regions of PRR37. Our study provides new insights into the role of CK-mediated phosphorylation of PRRs in LD-dependent floral repression in rice and in other plants.

**MATERIALS AND METHODS**

Plasmid construction and recombinant protein production

The cDNAs of Hd6, PRR37 and partial region of OsLHY were obtained by RT-PCR with total RNA extracted from the leaves of the japonica-indica hybrid rice cultivar ‘Milyang23’ using the gene-specific primers (Supplementary Table S1). The Hd6 and PRR37 were ligated into the plCB8/GW/TOPO plasmid (Invitrogen, USA). Then, the Hd6 and PRR37 DNA fragments in plasmids were digested with EcoRI. For expression in E. coli, the fragments were inserted into pGEX-4T-1 for glutathione S-transferase (GST)-tagged Hd6/CK2α protein (GST-Ck2α) and the modified pET28a for His(6x)-Maltose binding protein (HisMBP)-tagged PRR37 protein (HisMBP-PRR37) and HisMBP-LHYC (hC-terminal region (520-719 residues) of OsLHY; Ogiso et al., 2010). Proteins were expressed in Rosetta 2 cells after induction by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in SOB liquid media for 16 h at 20°C. After spin-down to remove the SOB media, cells were broken by sonication, and the tagged proteins were purified with GST-Bind agarose resin (Ebisu Biotech, Korea) or His-Bind agarose resin (Ebisu Biotech, Korea) according to the manufacturers' instructions. The GST-tag was removed from eluted GST-CK2α fusion protein by biotinylated thrombin protease (Merck, Germany) for in vitro kinase assay. To remove the thrombin, GST-Ck2α eluate was incubated with streptavidin agarose (Merck, Germany) for 30 min at room temperature. The eluates were concentrated using Amicon Ultra (Millipore, Germany). The cDNA of EL1 was acquired from total RNA in leaf blades of rice cultivar ‘Nipponbare’. Recombinant His(6x)-tagged EL1 CKI protein (His(CKI)) was expressed as described earlier (Kwon et al., 2014).

In vitro pull-down assay

Eluted HisMBP proteins (a negative control) and HisMBP-PRR37 were incubated with GST-Ck2α or His-CKI in GST-Bind Agarose Resin (Ebisu Biotech, Korea) or MBP-Bind Agarose Resin (Ebisu Biotech, Korea), respectively, at 4°C for 1 h. The resin was washed four times in GST pull-down washing buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM β-mercaptoethanol, 1% Triton X-100 and 0.2% glycerol) or MBP pull-down washing buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA). To boil the proteins bound to the resin, 5X SDS-PAGE loading buffer was added and heated at 100°C for 4 min. Proteins were resolved by SDS-PAGE and immunoblotted using antibodies against GST (Santa Cruz Biotechnology, USA), MBP (Santa Cruz Biotechnology, USA), and His(6x)-tag (Abcam, USA).
Subcellular localization and bimolecular fluorescence complementation (BiFC) assays

For YFP-tagged and partial YFP-tagged PRR37 constructs, PCR-amplified PRR37 cDNA was ligated into pCS8/GW/TOPO plasmid (Invitrogen, USA). Each cDNA of PRR37, EL1 or Hd6 was cloned into the vector with CaMV 35S promoter: YFP (pEarlyGate101 or pEarlyGate104) and the BIFC Gateway vectors to examine their subcellular localization and in vivo interaction (Citovsky et al., 2006). PRR37 clones were fused into four BIFC plasmid sets: pSAT5-DEST-cEYFP(175-end)-C1(B) (pE3130), pSAT5(A)-DEST-cEYFP(175-end)-N1 (pE3132), pSAT4(A)-DEST-nEYFP(1-174)-N1 (pE3134), and pSAT4-DEST-nEYFP(1-174)-C1 (pE3136), to generate cYFP-PRR37, PRR37-cYFP, PRR37-nYFP, and nYFP-PRR37, respectively. The same method was used for partial YFP-tagged EL1/CKI and Hd6/CK2α constructs. Each pair of recombinant plasmids encoding nEYFP and cEYFP fusions was mixed 1:1 (w/w), co-bombarded into onion epidermal layers using a DNA particle delivery system (Biolistic PDS-1000/He, BioRad, USA), and incubated on 0.5x Murashige and Skoog (MS) solid media in the presence or absence of MG132 (50 μM) for 16-24 h at 22°C under light or dark incubation, followed by observation and image analysis using a Confocal Laser Scanning Microscope II (LSM710, Carl Zeiss, Germany).

In vitro kinase assay

To do the in vitro kinase assay, a 50 μl reaction containing approximately 1.0 μg/μl of the recombinant kinase (cCK2α or His-CKI) and 1.5 μg/μl of the substrate in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 10 mM MgAc, 2 mM HEPES, 0.1 mM ATP, 2.5 mM MgCl2, and 10 μCi [γ-32P]ATP (Izotop, Hungary) was incubated at 30°C for 30 min. To stop the reaction, 5x SDS-PAGE loading buffer was added. After the separation of proteins by SDS-PAGE, the 32P-labeled proteins were visualized by autoradiography. This assay was modified from previous research (Kang et al., 2013; Youn et al., 2013)

RESULTS

CK2α and CKI interact with PRR37 in vitro

The suppression of flowering by Hd6 and Hd16 in non-inductive LD conditions requires functional Hd2 (Hori et al., 2013; Shibaya et al., 2011; Yamamoto et al., 2000), indicating that the floral repressor Hd2 acts downstream of Hd6 and Hd16 in the same pathway regulating rice flowering. Hd2, Hd6, and Hd16 encode PRR37, CK2α, and CKI, respectively (Hori et al., 2013; Koo et al., 2013; Takahashi et al., 2001). Also, in Arabidopsis, PRR7 is phosphorylated by an as-yet unknown protein kinase(s) (Fujiwara et al., 2008). Thus, we hypothesized that the genetic interactions of Hd2, Hd6, and Hd16 may involve the phosphorylation of PRR37 by CK2α or CKI, via direct interaction. To examine this hypothesis, we first performed in vitro pull-down assays to examine the interactions of CK2α-PRR37 and CKI-PRR37. To this end, we purified four recombinant fusion proteins, the N-terminal GST-tagged CK2α (GST-CK2α), GST (a negative control), His(6x)-MBP-tagged PRR37 (HisMBP-PRR37), and HisMBP (a negative control) (see “Materials and Methods”). When GST-CK2α was pulled down with anti-GST antibody-conjugated agarose resin, HisMBP-PRR37 co-immunoprecipitated, but not the negative control HisMBP (Fig. 1A), indicating that PRR37 directly interacts with CK2α in vitro. Furthermore, when HisMBP-PRR37 was pulled down with anti-MBP antibody-conjugated agarose resin, His-CKI also co-immunoprecipitated with HisMBP-PRR37, but not with HisMBP.
CKI and CK2α Phosphorylate OsPRR37
Choon-Tak Kwon et al.

rescence in the nucleus, as did cells co-expressing nYFP-CKI and PRR37-cYFP (Fig. 2B), indicating that CK2α and CKI directly interact with PRR37 in the nucleus.

**CK2α and CKI phosphorylate PRR37**

CK2α and CKI can phosphorylate specific substrates; CK2α phosphorlases OsLHY in the circadian clock and flowering regulation (Ogiso et al., 2010), and CKI phosphorylates SLR1 in GA signaling and Ghd7 in flowering regulation (Dai and Xue, 2010; Hori et al., 2013). To examine whether these two protein kinases can phosphorylate PRR37, we conducted in vitro kinase assays with HistMBP-PRR37, GST-CK2α, and His-CKI, using [γ-32P]ATP as the label. For the GST-CK2α fusion protein, we used a bionylated thrombin protease to remove the GST-tag before conducting the kinase assay. For in vitro kinase assays, we confirmed that recombinant CK2α (rCK2α) phosphorylated the positive control HistMBP-LHYc, the C-terminal region (520-719 residues) of OsLHY (Ogiso et al., 2010) (Fig. 3A). The kinase assays revealed that rCK2α (Fig. 3A) and His-CKI (Fig. 3A), can phosphorylate HistMBP-PRR37, but do not phosphorylate the negative control HistMBP. His-CKI also auto-phosphorylated, as previously reported (Kwon et al., 2014) (Fig. 3B).

To determine the phosphorylation sites in PRR37, we first examined the PRR37 sequence with a casein kinase-specific phosphorylation site prediction algorithm, KinasePhos 2.0 (http://kinasephos2.mbc.nctu.edu.tw/). KinasePhos predicted 46 phosphorylated amino acids (aa) in PRR37, 36 serines and 10 threonines (Fig. 4A and Supplementary Fig. S1). To investigate the region of PRR37 phosphorylated by CK2α and/or CKI, we divided PRR37 (742 total residues) into three segments, the N-terminal (PRR37n; aa 1-200, including the Pseudo Receiver [PR] domain), middle (PRR37m; aa 201-480), and the C-terminal segments (PRR37c; aa 481-742, including the CONSTANS, CO-like, and TOC1 [CCT] domain) (Fig. 4A). We fused these three segments of PRR37 to the N-terminal HistMBP-tag and purified the fusion proteins from E. coli. In vitro kinase assays revealed that CK2α and CKI both phosphorylated PRR37c, which includes the CCT domain. Also, neither CK2α nor CKI phosphorylated PRR37n, which includes the PR domain (Fig. 4B). These data suggest that CK2α and CKI phosphorylate different residues of PRR37, which likely affects the activity and stability of PRR37, similar to Arabidopsis PRR proteins (Fujiiwara et al., 2008).

**DISCUSSION**

Arabidopsis PRR7, an ortholog of rice PRR37, functions as an important component of the circadian clock (Nakamichi et al., 2005; 2007; Salome and McClung, 2005). In monocot plants, such as rice, wheat, barley, and sorghum, natural mutations of PRR7 orthologs affect seasonal or regional adaptation by modulating photoperiod sensitivity and flowering time, to maximize plant survival and grain yield (Beales et al., 2007; Koo et al., 2013; Murphy et al., 2011; Turner et al., 2005). The Arabidopsis *prn*7 loss-of-function mutants flower slightly late in inductive LD conditions, but the rice *prn*7 knockout mutants flower earlier in non-inductive LD conditions (Koo et al., 2013; Nakamichi et al., 2007). These results suggest that the regulatory roles of the PRR7 orthologs in growth and development have diverged in monocot and dicot plants.

In Arabidopsis, posttranslational modification of circadian clock components, such as CCA1 and PRR proteins (PRR1/TOC1,
CKI and CK2 phosphorylate OsPRR37
Choon-Tak Kwon et al.

Fig. 3. CK2α and CKI phosphorylates PRR37. (A) In vitro kinase assay using recombinant CK2α (rCK2α) showed phosphorylation of HisMBP-LHYc [a positive control; C-terminal region (aa 520-719) of OsLHY; Ogiso et al., 2010] and HisMBP-PRR37 proteins. HisMBP was used as a negative control. (B) In vitro kinase assay showed phosphorylation of HisMBP-PRR37 by His-CKI and auto-phosphorylation of His-CKI. Input of each protein was shown by Coomassie blue staining (upper panels), and phosphorylated proteins were detected by [γ-32P]ATP autoradiography (lower panels). The black triangles above the panels indicate concentrations of substrates. These results were reproduced at least two times with the same results.

PRR3, PRR5, PRR7, and PRR9), influences protein activity and stability, via phosphorylation and selective proteolysis (Fujisawa et al., 2008). Phosphorylated TOC1 and PRR5 strongly interact with the F-box protein ZEITLUPE (ZTL), indicating that their regulation by proteolysis modulates circadian rhythm (Fujisawa et al., 2008). Moreover, phosphorylated PRR5 promotes the phosphorylation and nuclear accumulation of TOC1 (Wang et al., 2010). Nevertheless, the protein kinase(s) responsible for phosphorylating PRR proteins have not yet been identified. In this study, we show that in rice, the protein kinases CKI and CK2α directly modify PRR37 at the posttranslational level (Figs. 1A, 2A, and 3A).

In floral repression under non-inductive LD conditions, Hd6/CK2α may phosphorylate downstream LD-dependent floral repressor(s). Several studies have reported the epistatic interactions between Hd2/PRR37 and other flowering-time regulators in rice. First, genetic studies showed that Hd6 acts upstream of Hd2 to delay flowering time, because hd2 is epistatic to Hd6 or hd6 in LD conditions (Yamamoto et al., 2000). Thus, we postulate that CK2α regulates PRR37 at the posttranslational level, because CK2α interacts with and phosphorylates PRR37 (Figs. 1A, 2A, and 3A). Ogiso et al. (2010) reported that the LD-dependent floral repression of Hd6 requires functional Hd1 because hd1 is epistatic to Hd6 or hd6 in LD conditions, but CK2α does not interact with or phosphorylate Hd1. Thus, they speculated that Hd1 activity may be regulated by an unknown regulatory protein phosphorylated by CK2α; we postulate that PRR37 is a strong candidate for this unknown protein. Lin et al. (2000) reported that Hd1 genetically acts downstream of Hd2 to delay flowering time in LD, but no work has yet reported a direct link between these two CCT-domain containing proteins, Hd1 and PRR37, at the transcriptional or posttranslational level. For this reason, further work should examine the biochemical relationships of Hd1, PRR37, and CK2α to reveal the molecular mechanism of Hd6-mediated floral repression in LD conditions. In addition, it would be worthwhile to examine whether CK2α, CKI, or both can interact
with other OsPRR proteins such as OsTOC1/OsPRR1, OsPRR59, OsPRR73, and OsPRR95.

Second, Hd16 also acts as an LD-dependent floral repressor by downregulating Ehd1 expression (Hori et al., 2013; Kwon et al., 2014). Hori et al., (2013) used in vitro kinase assays to show that CKI interacts with and phosphorylates the LD-dependent floral repressor Ghd7, but does not phosphorylate Hd1. This suggests that CKI downregulates Ehd1 expression by upregulating Ghd7 activity at the posttranslational level. Similar to Hd6, Hd16 requires functional Hd2 to delay flowering in LD (Hori et al., 2013; Shibaya et al., 2011; Yamamamoto et al., 2000), indicating that Hd6, Hd16, and Hd2 function in the same genetic pathway of LD-dependent floral repression. Indeed, we found that CKI also interacts with and phosphorylates PRR37 (Figs. 1B, 2B, and 3B). These results strongly suggest that the posttranslational modification of PRR37 by CKI and CK2α likely affects its activity and stability, which should be determined in vivo.

It is noteworthy that CKI and CK2α phosphorylate distinct regions in the PRR37 protein; CKI phosphorylates the recombinant partial proteins PRR37m and PRR37c (Fig. 4B), but CK2α phosphorylates only PRR37m (Fig. 4B). This suggests that the levels of phosphorylation by CKI and CK2α might separately regulate the activity and/or stability of PRR37. Further in vitro kinase assays combined with site-specific mutagenesis of PRR37 will be necessary to identify the exact sites where CKI and CK2α phosphorylate PRR37.

Natural variants of Hd1, PRR37, Ghd7, DTH8, Hd6, and Hd16 occur in the rice varieties that are currently cultivated in Asia and Europe, and these variants play important roles in the downregulation of Ehd1 expression to delay flowering in natural LD conditions (Fig. 5). Hd16/CKI inhibits flowering in the Ehd1-related pathway through phosphorylation of Ghd7 (Hori et al., 2013) and PRR37. However, the relationship between PRR37 and Ehd1 remains unclear, based on two conflicting reports (Koo et al., 2013; Yan et al., 2013). Hd6/CK2α might phosphorylate PRR37 to downregulate Hd3a and RFT1 expression in the Hd1-related pathway (Ogiso et al., 2010) by as-yet-unknown mechanisms. In addition, CK2α phosphorylates OsLHY in vitro, although Hd6/CK2α is not involved in the circadian rhythm in rice (Ogiso et al., 2010). The LD-dependent repression of flowering by Ghd7 and PRR37 is genetically additive (Kim et al., 2013; Koo et al., 2013), indicating that both act independently and synergistically. DTH8 genetically acts downstream of Hd16 because dth8 is epistatic to Hd16 (Hori et al., 2013), and thus it remains to be elucidated whether CKI directly interacts with and phosphorylates DTH8, an OsHAP3 subunit of the CCAAT-box-binding transcription factor (Wei et al., 2010). Currently, japonica rice cultivars can grow in high-latitude regions up to 53°N because they can flower extremely early under natural LD conditions (>14 h light/day) during the short summer period (Izawa 2007). Natural variations in the two major LD-dependent floral repressors PRR37 and Ghd7 are associated with seasonal and regional adaptation of rice to growth in the northernmost areas (Koo et al., 2013; Xue et al., 2008). In the northernmost rice cultivation regions, nonfunctional alleles of PRR37 and ghd7 are broadly distributed in rice cultivars, including ‘Iburiwase’, ‘Hoshinoyume’, and ‘H143’ (Fujino and Sekiguchi, 2005; Koo et al., 2013; Shibaya et al., 2011) (Supplementary Table S2). Notably, northern-limit rice cultivars have different combinations of Hd6 and Hd16 alleles. For example, Iburiwase has functional alleles of Hd6 and Hd16, Hoshinoyume has nonfunctional hd6 and functional Hd16 alleles, and H143 has functional Hd6 and nonfunctional Hd16 alleles (Fujino and Sekiguchi, 2005; Kwon et al., 2014; Nonoue et al., 2008; Shibaya et al., 2011) (Supplementary Table S2). However, these japonica rice cultivars exhibit the same early flowering time [about 75 days to heading (DTH)] under natural LD conditions in Suwon, Korea (37°N), strongly supporting the hypothesis that the effects of Hd6 and Hd16 on floral repression depend completely on functional PRR37 and Ghd7. Interestingly, an elite japonica rice cultivar ‘Koshihikari’ (about 105 DTH) has functional PRR37 and Ghd7, and nonfunctional hd6 and hd16 alleles, and thus flowers earlier than ‘Milyang23’ (about 117 DTH), which has functional PRR37, Ghd7, Hd6, and Hd16 alleles (Koo et al., 2013; Kwon et al., 2014; Matsubara et al., 2008a) (Supplementary Table S2). In the absence of CKI and CK2α functions, however, Koshihikari flowered much later than Iburiwase, Hoshinoyume, and H143 (105 vs. 75 DTH), but similar to the prr37-KO mutant in the ‘Dongjin’ background under LD (92 vs. 93 DTH) and SD (71 vs. 70 DTH) conditions (Koo et al., 2013). Although Koshihikari and Dongjin have different genetic backgrounds, it is notable that both japonica cultivars have been bred to grow in temperate regions and they have similar photoperiod sensitivities (21 vs. 23 days). Thus, it can be speculated that, at least in part, PRR37 may not be functional in Koshihikari, similar to the prr37-KO mutant.

In conclusion, we propose that CKI and CK2α may contribute to enhancing the photoperiod sensitivity of rice through phosphorylation of PRR37. Furthermore, our results provide new, important insights into CKI and/or CK2-mediated phosphorylation of PRR proteins in other plants including barley, wheat, sorghum, maize, and Arabidopsis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS
We thank Dr. Sangkhee Rhee at Seoul National University for donating the modified pET28a plasmid and Mr. Yong-Jae Kim.
at the National Center for Inter-University Research Facilities (NCIRF) for technical assistance in CLSM analysis. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2011-0017308).

REFERENCES

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichihoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science 309, 1052-1056.

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Mas, P., and Kay, S.A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science 293, 880-883.

Beales, J., Turner, A., Griffiths, S., Snape, J.W., and Laurie, D.A. (2007). A pseudo-response regulator is misexpressed in the photoperiod-insensitive Ppd-D1a mutant of wheat (Triticum aestivum L.). Theor. Appl. Genet. 113, 721-733.

Citovsky, V., Lee, L.Y., Sivas, G., Glick, E., Chen, M.H., Vainstein, A., Gafni, Y., Gelvin, S.B., and Tzfira, T. (2006). Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. J. Mol. Biol. 362, 1120-1131.

Corbesier, L., Vincent, C., Jang, S.H., Fornara, F., Park, J.W., Park, J.Y., Searle, P.A., Citovsky, V., Lee, L.Y., Sivas, G., Yamanouchi, U., and Yano, M. (2010). Adaptation of circadian clock entrainment in Arabidopsis through selective phosphorylation of a pseudo-response regulator. J. Biol. Chem. 285, 3292-3297.

Daniel, X., Sugano, S., and Tobin, E.M. (2004). CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in Arabidopsis. Proc. Natl. Acad. Sci. USA 101, 3292-3297.

Doi, K., Iwabuchi, M., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (2009). A pair of related genes with antagonistic roles in mediating flowering signals. Science 326, 1960-1962.

Kim, R.Y., Ikegami, A., Tamaki, Y., Suyama, M., and Shimamoto, K. (2008). Hd3a and RFT1 are essential for flowering in rice. Development 135, 767-774.

Kim, R.K., Yoko, S., and Shimamoto, K. (2009). A gene network for long-day flowering activates RFT1 encoding a mobile flowering signal in rice. Development 136; 3443-3450.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999). A genetic study of the Arabidopsis circadian clock. Plant Physiol. 121, 1960-1962.

Lin, H.X., Yamamoto, T., Sasaki, T., and Yano, M. (2000). Characterization and detection of epistatic interactions of 3 QTLs, Hd1, Hd2, and Hd3, controlling heading date in rice using nearly isogenic lines. Theor. Appl. Genet. 101, 1021-1028.

Matsubara, K., Kono, I., Sato, S., Kido, Y., and Mizuno, T. (2005). Adaptation of photoperiodic flowering signals in rice. Plant Physiol. 137, 1537-1545.

Matsubara, K., Kono, I., Horie, K., Nonoue, Y., Ono, N., Shimura, A., Mizubayashi, T., Yamamoto, S., Yamanoue, U., Shirasawa, K., et al. (2008a). Novel QTLs for photoperiodic flowering revealed by using reciprocal backcross inbred lines from crosses between japonica rice cultivars. Theor. Appl. Genet. 117, 935-945.

Matsubara, K., Yamanouchi, U., Wang, Z.X., Minobe, Y., Iwashita, T., and Yano, M. (2008b). Hdh2, a rice ortholog of the maize photoperiodic flowering marker gene Zvrn1, contributes to early flowering in japonica rice varieties. Mol. Plant. 1, 1960-1962.

Mizubayashi, T., Yamamoto, S., Yamanouchi, U., Shirasawa, K., et al. (2003). Adaptation of photoperiodic flowering signals in rice. Plant Physiol. 131, 1001-1009.

Mizuno, T. (2009). A genetic study of the Arabidopsis circadian clock. Plant Physiol. 149, 985-999.

Mizuno, T. (2005). Circadian-associated rice pseudo response regulator OsPRR37 regulates heading date and contributes to rice cultivation at a wide range of latitudes. Mol. Plant. 8, 1787-1796.

Murakami, M., Matushika, A., Ashikari, M., Yamashino, T., and Mizuno, T. (2005). Circadian-associated rice pseudo response regulators (OsPRRs): Insight into the control of flowering time. Mol. Cells. 20, 401-414.

http://molcells.org

Mol. Cells 87
Murakami, M., Tago, Y., Yamashino, T., and Mizuno, T. (2007). Characterization of the rice circadian clock-associated pseudo-kinase regulators in Arabidopsis thaliana. Biosci. Biotech. Biochem. 71, 1107-1110.

Murphy, R.L., Klein, R.R., Morishige, D.T., Brady, J.A., Rooney, W.L., Miller, F.R., Dugas, D.V., Klein, P.E., and Mullet, J.E. (2011). Coincident light and clock regulation of pseudoresponsive regulator protein 37 (PRR37) controls photoperiodic flowering in sorghum. Proc. Natl. Acad. Sci. USA 108, 16469-16474.

Nakamichi, N., Kita, M., Ito, S., Yamashino, T., and Mizuno, T. (2005). PSEUDO-RESPONSE REGULATORS, PRR9, PRR7 and PRR5, together play essential roles close to the circadian clock of Arabidopsis thaliana. Plant Cell Physiol. 46, 668-698.

Nakamichi, N., Kita, M., Niinuma, K., Ito, S., Yamashino, T., Mizoguchi, T., and Mizuno, T. (2007). Arabidopsis clock-associated pseudo-response regulators PRR9, PRR7 and PRR5, together play essential roles close to the circadian clock of Arabidopsis thaliana. Plant Physiol. 147, 154-165.

Nonoue, Y., Fujino, K., Hirayama, Y., Yamanouchi, U., Lin, S.Y., and Yano, M. (2008). Detection of quantitative trait loci controlling extremely early heading in rice. Theor. Appl. Genet. 116, 715-722.

Nusinow, D.A., Helfer, A., Hamilton, E.E., King, J.J., Imaizumi, T., Schultz, T.F., Faire, E.M., and Kay, S.A. (2011). The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl photomorphogenesis. Plant Cell Physiol. 52, 808-820.

Park, S.H., Miller, F.R., Dugas, D.V., Klein, P.E., and Mullet, J.E. (2003). Comparative genetic studies on the APRR5 and APRR7 genes belonging to the APRR1/TOC1 quintet implicated in circadian rhythm, control of flowering time, and early photomorphogenesis. Plant Cell Physiol. 44, 1119-1130.

Park, S.W., Xiong, Y., Inoue, H., Zhang, W., Zhou, L., and Wang, C.R. (2013). A major QTL, Ghd8, plays pleiotropic roles in regulating grain productivity, plant height, and heading date in rice. Mol. Plant 6, 319-330.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.

Song, Y.H., Smith, P.R., To, J.J., Millar, A.J., and Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. Science 336, 1045-1049.