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

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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 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 photoperiod pathway for floral induction (Huq et al., 2000; Kardailsky et al., 1999; Kobayashi et al., 1999; Park et al., 1999). The florigen 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 florigens 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., 2008b; 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 upstream 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

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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-ELF3-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 between 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 bluelight signaling and circadian rhythm by decreasing the stability of cryptochrome 2 through phosphorylation (Tan et al., 2013). Moreover, the highly conserved circadian clock component CK2 positively regulates the stability of the clock oscillators CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) (Daniel et al., 2004; Lu et al., 2011; Portoles and Mas 2010; Sugano et al., 1998) and rice LATE ELONGATED HYPOCOTYL (OsLHY) (Ogiso et al., 2010). Rice *Hd6*, which encodes the α subunit of CK2 (CK2α), does not control circadian rhythm but delays flowering time in LD conditions in the presence of functional *Hd1*, indicating that *Hd6* genetically acts upstream of *Hd1* in LD-dependent floral repression (Ogiso et al., 2010). However, CK2α does not interact with or phosphorylate Hd1 (Ogiso et al., 2010). Also, *Hd16*/*Early flowering1* (*EL1*), which encodes CKI, not only represses gibberellin signaling by phosphorylating SLENDER RICE1 (SLR1) but also inhibits LD-dependent flowering by phosphorylating Ghd7, resulting in the suppression of *Ehd1*, *Hd3a*, and *RFT1* (Dai and Xue, 2010; Hori et al., 2013; Kwon et al., 2014).

Pseudo-Response Regulators (PRRs) are essential circadian clock components in *Arabidopsis* (Alabadi et al., 2001; Farre and Kay, 2007; Ito et al., 2009; Kaczorowski and Quail, 2003; Nakamichi et al., 2007; Yamamoto et al., 2003). The five *PRR* genes (*PRR1*/*TIMING OF CAB EXPRESSION 1* [*TOC1*], *PRR3*, *PRR5*, *PRR7*, and *PRR9*) show differential circadian expression from dawn to dusk (Matsushika et al., 2000). *In planta*, all of the PRRs are phosphorylated, which may be essential for their function. However, the protein kinases responsible for the phosphorylation of PRRs have not yet been identified (Fujiwara et al., 2008). Rice also has five orthologs of *Arabidopsis PRR* genes: *OsPRR1*/*OsTOC1*, *OsPRR37*, *OsPRR59*, *OsPRR73,* and *OsPRR95* (Murakami et al., 2005; 2007). The rice ortholog of *Arabidopsis PRR7*, *Hd2*/*OsPRR37* (hereafter *PRR37*) functions as one of 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 pCR8/GW/TOPO plasmid (Invitrogen, USA). Then, the *Hd6* and *PRR37* DNA fragments in plasmids were digested with *Eco*RI. 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 [C-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 (Elpis Biotech, Korea) or His-Bind agarose resin (Elpis 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 (Elpis Biotech, Korea) or MBP-Bind Agarose Resin (Elpis 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 pCR8/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 (rCK2α 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 $MgCl_2$and 10 μCi [γ-^{32}P]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 ^{32}P-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 resins, 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 resins, His-CKI also co-immunoprecipitated with HisMBP-PRR37, but not with HisMBP

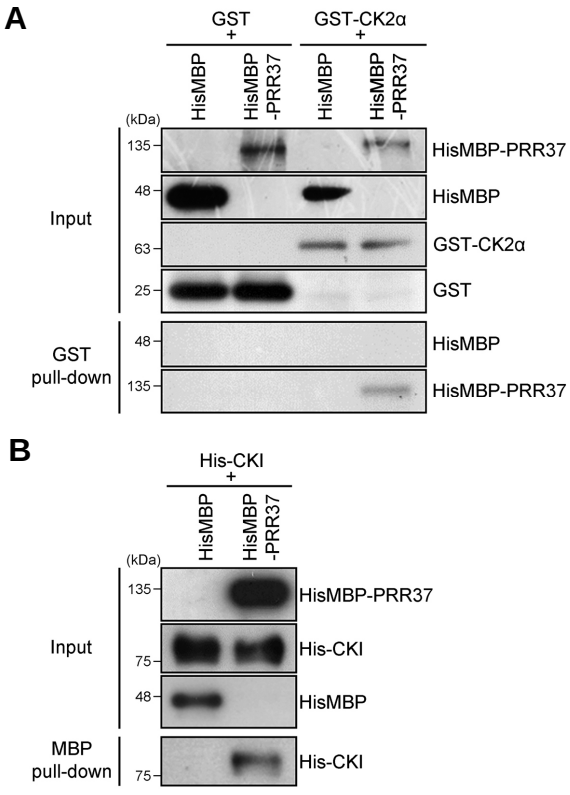

Fig. 1. PRR37 physically interacts with CK2α and CKI *in vitro*. (A) Interaction between GST-CK2α and HisMBP-PRR37 by *in vitro* pull-down assay using GST-Bind agarose resin. GST and GST-CK2α were detected using anti-GST antibody. HisMBP and HisMBP-PRR37 (input) were detected by anti-His antibody. After GST pull-down, HisMBP and HisMBP-PRR37 were detected by anti-MBP antibody. (B) His-CKI and HisMBP-PRR37 co-immunoprecipitated using MBP-Bind agarose resin. After MBP pull-down, His-CKI was detected by anti-His antibody. These experiments were repeated at least twice with the same results.

(Fig. 1B). These results show that Hd6/CK2α and Hd16/CKI likely interact with Hd2/PRR37 for floral repression.

CK2α and CKI interact with PRR37 *in vivo*

To examine their potential interactions *in vivo*, we first examined the subcellular localization of CK2α and PRR37, as previous work showed that CKI localizes to the nucleus (Dai and Xue, 2010). To examine the subcellular localization of CK2α and PRR37, we constructed C-terminal yellow fluorescent protein (YFP)-tagged CK2α (CK2α-YFP) and N-terminal YFP-tagged PRR37 (YFP-PRR37) and bombarded these plasmids into onion epidermal cell layers. In these cells, we observed strong YFP signals in the nuclei expressing the CK2α-YFP and YFP-PRR37 proteins (Fig. 2A), indicating that CKI, CK2α, and PRR37 all localize to the nucleus.

To confirm the interactions of CK2α-PRR37 and CKI-PRR37 *in vivo*, we performed bimolecular fluorescence complementation (BiFC) assays. In the negative control experiment, we detected no YFP fluorescence in cells co-expressing nYFP and cYFP-PRR37 plasmids (Fig. 2B). In the positive control experiment, we observed YFP signals as speckles in the nucleus in cells co-expressing the known interactors nYFP-ELF3 and

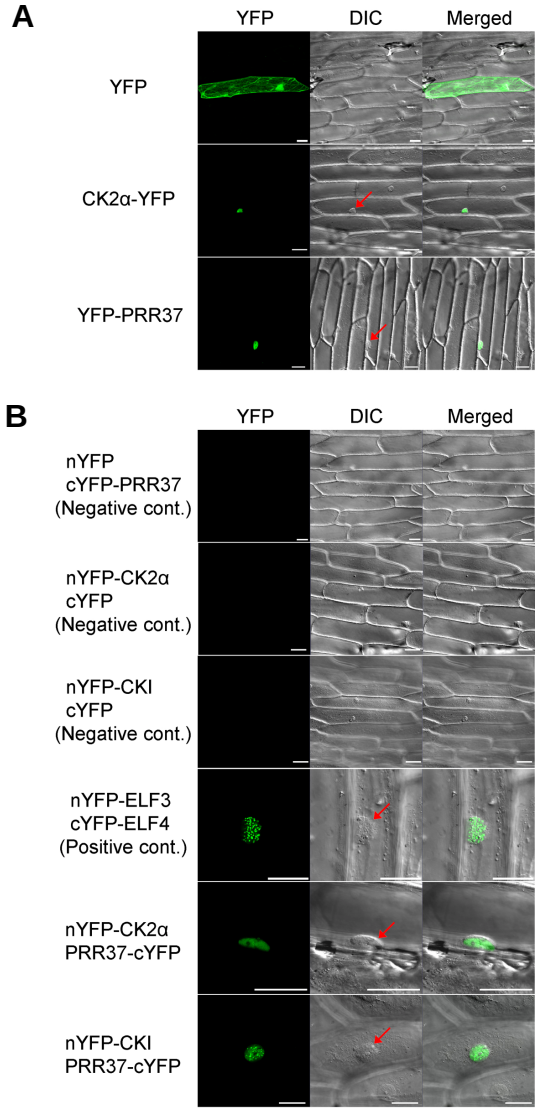

Fig. 2. PRR37 interacts with both CK2α and CKI *in vivo*. (A) Subcellular localization of CK2α-YFP and YFP-PRR37 in onion epidermal cells. The panels show YFP, differential interference contrast (DIC) and merged images of onion epidermal cells. Empty vector (pEarlygate101) was used as the YFP control. Fluorescent signals of CK2α-YFP and YFP-PRR37 appeared in the nucleus. (B) Empty BiFC plasmids (nYFP and cYFP-PRR37; nYFP- CK2α and cYFP; nYFP-CKI and cYFP) were used as a negative control. The YFP signals of co-expressed nYFP-AtELF3 and cYFP-AtELF4 (a positive control; At, *Arabidopsis thaliana*) were detected in the nucleus, as described (Herrero et al., 2012). For CK2α-PRR37 and CKI-PRR37 interactions, nYFP-CK2α and nYFP-CKI were co-bombarded with PRR37-cYFP. BiFC analysis by transiently expressing nYFP-CK2α and nYFP-CKI with PRR37-cYFP indicated the interaction of nYFP-CK2α and nYFP-CKI with PRR37-cYFP in nucleus. Red arrows indicate the nucleus. These experiments were repeated at least three times with the same results. Scale bar = 50 μm.

cYFP-ELF4 (Herrero et al., 2012; Nusinow et al., 2011) (Fig. 2B). Finally, we found that the onion epidermal cells co-expressing nYFP-CK2α and PRR37-cYFP showed YFP fluorescence 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 phosphorylates 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 HisMBP-PRR37, GST-CK2α, and His-CKI, using [γ-^{32}P]ATP as the label. For the GST-CK2α fusion protein, we used a biotinylated 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 HisMBP-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 HisMBP-PRR37, but do not phosphorylate the negative control HisMBP. His-CKI also autophosphorylated, 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 HisMBP-tag and purified the fusion proteins from *E. coli*. *In vitro* kinase assays revealed that CK2α and CKI both phosphorylated PRR37m (Fig. 4B). However, only CKI 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 (Fujiwara 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 *PRR37* 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 prr7* loss-of-function mutants flower slightly late in inductive LD conditions, but the rice *prr37* knockout mutants flower early in non-inductive LD conditions (Koo et al., 2013; Nakamichi et al., 2007). These results suggest that the regulatory roles of the *PRR37* 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,

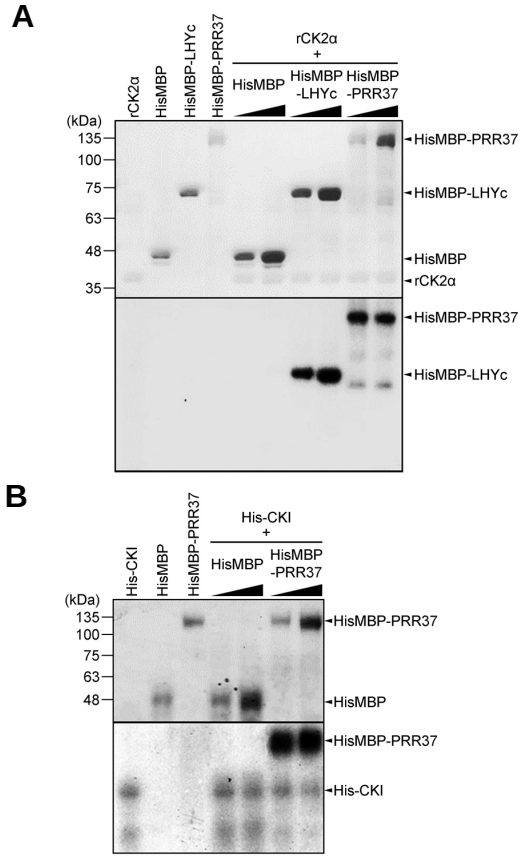

Fig. 3. CK2α and CKI phosphorylates PRR37. (A) *In vitro* kinase assay using recombinant CK2α (rCK2α) showed phosphorylation of HisMBP-LHYc [a positive control; C-terminal region (aa 520-719) of OsLHY; Ogiso et al., 2010] and HisMBP-PRR37 proteins. HisMBP was used as a negative control. (B) *In vitro* kinase assay showed phosphorylation of HisMBP-PRR37 by His-CKI and autophosphorylation of His-CKI. Input of each protein was shown by Coomassie blue staining (upper panels), and phosphorylated proteins were detected by [γ-^{32}P]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.

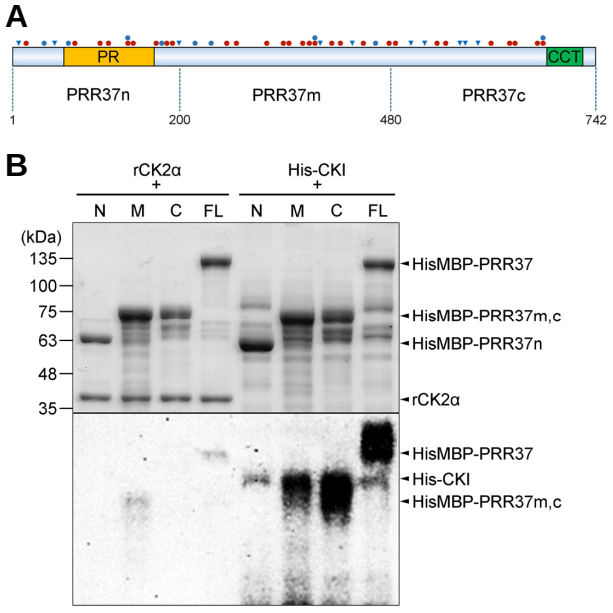

Fig. 4. CK2α and CKI phosphorylate specific regions of PRR37. (A) The protein structure and predicted phosphorylation sites of PRR37 from rice cultivar 'Milyang23'. PR in yellow indicates a pseudo receiver (PR) domain and CCT in green indicates a CONSTANS, CO-like, and TOC1 (CCT) domain. The three partial PRR37 proteins were: PRR37n (1-200 aa), PRR37m (201-480 aa) and PRR37c (481-742 aa). Circles represent possible phosphorylation sites at serine by CKI (red) and CK2 (blue) residues. Inverted triangles indicate possible phosphorylation sites at threonine by CKI (red) and CK2 (blue). The phosphorylation prediction was obtained from KinasePhos 2.0 (http://kinasephos2.mbc.nctu.edu.tw/). (B) *In vitro* kinase assay of PRR37n (N), PRR37m (M), PRR37c (C) and full-length PRR37 (FL) proteins by rCK2α and His-CKI. PRR37m and PRR37 were phosphorylated by rCK2α. Input of each protein was shown by Coomassie blue staining (upper panels), and phosphorylated proteins were detected by [γ-^{32}P]ATP autoradiography (lower panels). PRR37m, PRR37c and PRR37 were phosphorylated by His-CKI. Self-phosphorylation of His-CKI was also detected. These experiments were repeated at least three times with the same results.

PRR3, PRR5, PRR7, and PRR9), influences protein activity and stability, *via* phosphorylation and selective proteolysis (Fujiwara 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 (Fujiwara 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. 1, 2, and 3).

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 (Yammamoto 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

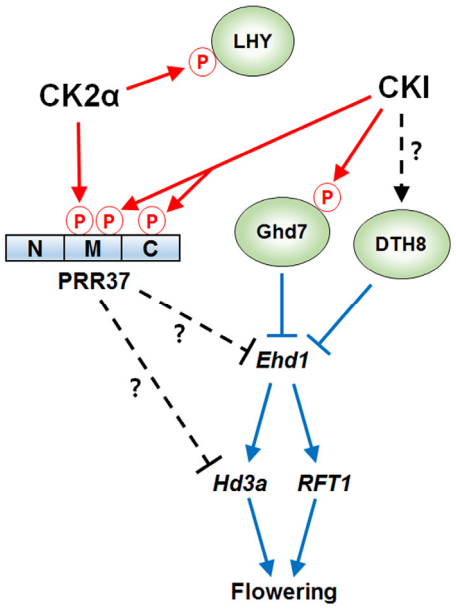

Fig. 5. Schematic model of regulation of PRR37 and other floral integrators by CKI and CK2α, in photoperiodic flowering under long-day conditions in rice. Blue and red arrows indicate the regulation at the transcriptional and posttranslational levels, respectively.

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; Yammamoto 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).

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