Natural alleles of CIRCADIAN CLOCK ASSOCIATED1 contribute to rice cultivation by fine-tuning flowering time

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Abstract

The timing of flowering is a crucial factor for successful grain production at a wide range of latitudes. Domestication of rice (Oryza sativa) included selection for natural alleles of flowering-time genes that allow rice plants to adapt to broad geographic areas. Here, we describe the role of natural alleles of CIRCADIAN CLOCK ASSOCIATED1 (OsCCA1) in cultivated rice based on analysis of single-nucleotide polymorphisms deposited in the International Rice Genebank Collection Information System database. Rice varieties harboring japonica-type OsCCA1 alleles (OsCCA1a haplotype) flowered earlier than those harboring indica-type OsCCA1 alleles (OsCCA1d haplotype). In the japonica cultivar “Dongjin”, a T-DNA insertion in OsCCA1a resulted in late flowering under long-day and short-day conditions, indicating that OsCCA1 is a floral inducer. Reverse transcription quantitative PCR analysis showed that the loss of OsCCA1a function induces the expression of the floral repressors PSEUDO-RESPONSE REGULATOR 37 (OsPRR37) and Days to Heading 8 (DTH8), followed by repression of the Early heading date 1 (Ehd1)–Heading date 3a (Hd3a)–RICE FLOWERING LOCUS T 1 (RFT1) pathway. Binding affinity assays indicated that OsCCA1 binds to the promoter regions of OsPRR37 and DTH8. Naturally occurring OsCCA1 alleles are evolutionarily conserved in cultivated rice (O. sativa). Oryza rufipogon-I (Or-I) and Or-III type accessions, representing the ancestors of O. sativa indica and japonica, harbored indica- and japonica-type OsCCA1 alleles, respectively. Taken together, our results demonstrate that OsCCA1 is a likely domestication locus that has contributed to the geographic adaptation and expansion of cultivated rice.

Introduction

Flowering time is crucial for regional and seasonal adaptation of plants. In cultivated rice (Oryza sativa), flowering time (also called heading) is closely related to grain yield and is thus of great agronomic importance. Rice is a facultative short-day (SD) plant whose flowering is promoted under SD conditions and is inhibited under long-day (LD) conditions. Rice varieties cultivated in the tropics can...
complete two or even three harvests each year (double- or triple-cropping) due to the shorter photoperiod and longer warm season compared to more temperate regions (Lin et al., 2021). However, since seasonal temperatures in temperate regions limit the rice cultivation period from late spring to early autumn, rice needs to flower during summer days, which are typical LD conditions, to reach the grain-filling stage before the onset of cold weather (Fujino et al., 2019). Thus, a weaker photoperiod sensitivity is a critical factor for rice adaptation to high-latitude regions.

Many flowering genes have been identified as quantitative trait loci for heading date in crosses between japonica and indica cultivars with distinct heading dates and shown to encode floral regulators that allow rice to flower early in temperate regions (Yamamoto et al., 2000; Yano et al., 2000; Fujino et al., 2013; Li et al., 2015; Han et al., 2017; Zhang et al., 2018). Among these regulators, floral repressors such as Heading date 1 (Hd1), Grain number, plant height, and heading date 7 (Ghd7, also named Hd4), Days to Heading 8 (DTH8, also named Ghd8 and Hd5), and rice PSEUDO-RESPONSE REGULATOR 37 (OsPRR37, also named Hd2 and DTH7) (Xue et al., 2008; Wei et al., 2010; Koo et al., 2013; Nemoto et al., 2016) reduce the expression of the downstream florigen genes and delay flowering time under LD conditions. Importantly, loss-of-function alleles in these floral repressors raise the expression levels of florigen genes, leading to promotion of flowering even under non-inductive daylength conditions. Thus, these natural alleles constitute a useful genetic reservoir for engineering rice varieties amenable to cultivation in high-latitude regions (Goretti et al., 2017; Zhang et al., 2019).

Hd1, a rice ortholog of the Arabidopsis (Arabidopsis thaliana) floral activator CONSTANS (CO), has opposite effects on Heading date 3a (Hd3a) expression depending on daylength (Yano et al., 2000; Nemoto et al., 2016; Zhang et al., 2017). Hd1 promotes flowering under SDs by activating Hd3a transcription but represses flowering under LDs by suppressing Hd3a transcription. Therefore, rice plants carrying hd1 loss-of-function alleles can be cultivated in high-latitude regions because of the de-repression of Hd3a, which allows flowering under a non-inductive photoperiod (Takahashi et al., 2009; Wu et al., 2020).

In addition to the conserved Hd1 pathway, rice has a unique Early heading date 1 (Ehd1) pathway that activates the expression of the two rice orthologs of Arabidopsis FLOWERING LOCUS T (FT), Hd3a, and RICE FLOWERING LOCUS T 1 (RFT1), under SDs and LDs, respectively (Doi, 2004; Cho et al., 2016; Nemoto et al., 2016). Ghd7 encodes a protein with a CO, CO-LIKE, TOC1 (CCT) domain and Ghd7 transcript levels are high in LDs (Xue et al., 2008). Under LD conditions, a peak in Ghd7 abundance in the morning is sufficient to repress Ehd1 transcription, resulting in delayed flowering. DTH8 encodes a putative HEME ACTIVATOR PROTEIN 3 subunit of the CCAAT-box-binding transcription factor (TF) complex and represses Ehd1 (Wei et al., 2010; Wang et al., 2019). The chromosome segment substitution line 61 (CSSL61) carrying a nonfunctional dth8 allele exhibits early flowering in LDs as a consequence of reduced photoperiod sensitivity.

The components of the circadian clock that are conserved between Arabidopsis and rice are integrated into the photoperiodic flowering network (Song et al., 2015). Among them, we previously showed that rice OsPRR37, which is orthologous to Arabidopsis PRR7, promotes flowering under LD conditions by downregulating Ehd1 and Hd3a expression (Koo et al., 2013; Liu et al., 2018; Hu et al., 2021). Natural alleles with amino acid (aa) substitutions in the CCT domain of OsPRR37 promote flowering for adaptation to high-latitude regions (Koo et al., 2013). Furthermore, rice japonica cultivars harboring nonfunctional alleles of OsPRR37 and Ghd7 show early flowering at high latitudes. The effect of osprr37 is enhanced when combined with a defective ghd7 allele, leading to extremely early flowering in osprr37 ghd7 cultivars under LDs (Liu et al., 2015; Zhang et al., 2019).

In addition to loss-of-function alleles of floral repressors that promote flowering under non-inductive photoperiods, natural variation at loci encoding floral activators also contributes to regional adaptation of rice to higher latitudes. For instance, DTH2 and Ehd4 encode a CONSTANS-like protein and CCCH-type zinc finger protein, respectively (Gao et al., 2013; Wu et al., 2013). Strong functional alleles of DTH2 and Ehd4 have been intensively selected for adaptation to LD conditions during domestication, resulting in promotion of flowering by upregulation of Hd3a and RFT1.

In this study, we determined that rice CIRCADIAN CLOCK ASSOCIATED 1 (OsCCA1; LOC_Os08g06110) integrates rice flowering. In addition, we show that allelic variation at OsCCA1 contributes to the expansion of areas amenable for cultivation of rice varieties. In Arabidopsis, CCA1 encodes a core component of the circadian clock (Alabadí et al., 2002). CCA1 and LATE ELONGATED HYOCOTYL (LHY) encode MYB-type TFs with a single MYB DNA-binding domain, and are partially redundant, as the cca1 lhy double-mutant flowers much earlier than the cca1 single mutant (Mizoguchi et al., 2002). CCA1 and LHY are floral repressors that suppress GIGANTEA (GI) expression by directly binding to the GI promoter, resulting in delayed flowering time (Lu et al., 2012; Park et al., 2016). In rice, the single CCA/LHY ortholog OsCCA1 (also termed OsLHY) acts as an upstream regulator of the OsGI–Hd1 pathway to accelerate rice flowering (Sun et al., 2021). Here, we propose a possible regulatory mechanism by which OsCCA1 is involved in flowering time. Our results show that OsCCA1 activates the Ehd1–Hd3a/RFT1 pathway by suppressing the expression of OsPRR37 or DTH8 by directly or indirectly binding to their promoter regions. We also surveyed single-nucleotide polymorphisms (SNPs) in the OsCCA1 coding region that might be responsible for variation in flowering time in cultivated rice varieties (O. sativa) from the International Rice Genebank Collection Information System (IRGCIS) database. Notably, we discovered that rice varieties carrying japonica- and indica-type OsCCA1 alleles are cultivated in distinct geographical
latitudes from the northernmost regions to the Equator. Our study suggests that OsCCA1 alleles were crucial for the global adaptation of rice cultivation to a broad range of latitudes.

**Results**

**Characterization of OsCCA1**

We used the aa sequences of OsCCA1 and its putative orthologs to determine their relationship (Wang et al., 2011; Turner et al., 2013; Kusakina et al., 2015; Zhang et al., 2015; Cheng et al., 2019). The aa sequence alignments indicated that the single MYB DNA-binding domain of OsCCA1 is highly conserved across land plants (Supplemental Figure S1). Phylogenetic analysis revealed that OsCCA1 was more closely related to CCA1 sequences of monocotyledonous plants, including barley (Hordeum vulgare), wheat (Triticum aestivum), maize (Zea mays), and Brachypodium distachyon, than those of dicotyledonous plants including A. thaliana and soybean (Glycine max) (Supplemental Figure S2). This result was consistent with a previously reported phylogeny (Zhang et al., 2015).

The transcripts of flowering-time genes are most abundant in developing leaf tissues (Kojima et al., 2002; Tamaki et al., 2007; Komiya et al., 2008). We therefore investigated the expression of OsCCA1 in wild-type (WT; japonica rice cultivar “Hwayoung”) tissues grown in a paddy field under natural LD (NLD) conditions (>14 h light/day in Suwon, South Korea, 37°N latitude) at the vegetative stage (63 days after sowing [DAS]) and reproductive stage (118 DAS) by reverse transcription quantitative PCR (RT-qPCR). OsCCA1 transcripts accumulated to high levels in leaf sheaths, flag leaves, and leaf blades at both stages (Figure 1A), indicating that OsCCA1 transcripts are mainly abundant in leaves.

We also examined the diurnal expression pattern of OsCCA1 in the top two leaf blades collected from WT plants at 56 DAS under LDs (14.5-h light/9.5-h dark) or 28 DAS under SDs (10-h light/14-h dark). Similar to the rhythmic expression pattern seen for AtCCA1 (Alabadi et al., 2002; Mizoguchi et al., 2002), OsCCA1 showed a diurnal expression pattern with a peak at dawn in LDs (zeitgeber 1 [ZT1], 1 h after dawn) and SDs (ZT22) (Figure 1B and C).

We determined the subcellular localization of OsCCA1 by transiently transfecting onion epidermal cells with a construct encoding OsCCA1 fused to green fluorescent protein (GFP). Free GFP was evenly distributed throughout onion cells, but OsCCA1-GFP accumulated solely in the nucleus (Supplemental Figure S3A), indicating that OsCCA1 is a nuclear protein, consistent with other rice MYB TFs (Xiong et al., 2014).

Since AtCCA1 forms a homodimer upon its phosphorylation by CASEIN KINASE 2, which is necessary for maintaining the pace of the circadian clock (Daniel et al., 2004), we investigated whether OsCCA1 might also form a homodimer by yeast two-hybrid assays. Indeed, yeast colonies from the strain AH109 harboring the bait and prey vectors expressing full-length OsCCA1 cloned in-frame with the sequence of the GAL4 DNA binding and activation domain (AD) grew on selective medium (SD –Leu, –Trp, –His) and showed high β-galactosidase activity, indicating that OsCCA1 forms homodimers (Supplemental Figure S3B).

**OsCCA1 accelerates rice flowering**

To explore the role of OsCCA1 as a floral regulator, we obtained a T-DNA insertion mutant (oscca1-1, PFG_3D-03067) from the RiceGE database (http://signal.salk.edu/cgi-bin/RiceGE). The T-DNA was inserted into the eleventh exon of OsCCA1 (Supplemental Figure S4, A and B). We detected no OsCCA1 transcripts in the mutant by RT-PCR, indicating that oscca1-1 is a knockout mutant (Supplemental Figure S4C).

To investigate the flowering time of oscca1-1 and its parent japonica cultivar “Hwayoung” (hereafter referred to as WT), we grew rice plants in the paddy field under NLD conditions, or in growth chambers under SDs or LDs. The oscca1-1 mutant flowered about 11 days later (117 DTH) than the WT (106 DTH) under NLDs (Figure 2, A and B). The flowering times obtained from 4 years of field testing were generally similar with some variations (Supplemental Figure S5, A–C). Under SDs and LDs, oscca1-1 flowered around 18 and 19 days later (SD, 65 DTH; LD, 109) than WT (SD, 47 DTH; LD, 90 DTH), respectively (Figure 2, C and D). We also investigated the phenotypes of a second allele, oscca1-2, generated by CRISPR/Cas9-mediated genome
editing and carrying a 1-bp insertion introducing a premature stop codon (Supplemental Figure S6, A and B). As with oscca1-1, the oscca1-2 mutant flowered 9 days later than its parental japonica cultivar “Dongjin” (DJ) under NLD conditions (oscca1-2, 127 DTH; WT, 118 DTH) (Supplemental Figure S6, C and D). These observations indicated that OsCCA1 acts as a floral inducer.

To confirm the influence of OsCCA1 on flowering, we generated two independent transgenic rice lines overexpressing OsCCA1 (OsCCA1-OE1 and OE2). RT-qPCR analysis showed that OsCCA1 transcripts accumulate to about 15-fold higher levels in the overexpressing lines compared with the WT at ZT7, at which time OsCCA1 expression is low in the WT (Figure 2E). Overexpressing plants flowered 10 days earlier than their WT segregating siblings when grown in the paddy field under NLD conditions (Figure 2, F and G). The flowering time obtained from three independent T₃ (2019), T₄ (2020), and T₅ (2021) homozygous lines were generally similar with some variations (Figure 2, F and G and Supplemental Figure S5, D and E). Taken together, these results indicated that, in contrast to the molecular function of AtCCA1 as a floral repressor in Arabidopsis (Mizoguchi et al., 2002), OsCCA1 may induce flowering in rice.

Loss of OsCCA1 function perturbs the expression of flowering pathway genes
To elucidate the regulatory roles of OsCCA1 in floral induction, we determined the diurnal expression pattern of several genes regulating flowering in WT and the oscca1-1 mutant by RT-qPCR (Figure 3). Accordingly, we grew WT and oscca1-1 plants in growth chambers for 28 DAS in SD or 56 DAS in LD and harvested the penultimate leaf blades every 3 h over 24 h. The transcript levels of the rice florigen genes Hd3a and RFT1 were lower in oscca1-1 than in WT at dawn (ZT1) under both SD and LD conditions (Komiya et al.,
Transcript levels for \textit{Hd1} and \textit{Ehd1}, upstream regulators of \textit{Hd3a} and \textit{RFT1} (Izawa, 2002; Hayama et al., 2003; Itoh et al., 2010), were differentially affected by the loss of OsCCA1, with \textit{Hd1} expression being slightly higher in \textit{oscca1-1} compared with WT under both photoperiods (Figure 3, E and F), in agreement with a recent report (Sun et al., 2021). However, \textit{Ehd1} expression was much lower in \textit{oscca1-1} at night under both SD and LD conditions (Figure 3, G and H). These results indicate that OsCCA1 participates in the \textit{Ehd1}-mediated flowering induction pathway.

Previous studies have identified several regulators upstream of \textit{Ehd1}, including \textit{Ehd1} inducers (OsGI, OsMADS51, Ehd2, Ehd3, and Ehd4) (Kim et al., 2007; Matsubara et al., 2008, 2011; Gao et al., 2013; Lee and An, 2015) and \textit{Ehd1} repressors (Ghd7, OsPRR37, LEC2 and FUSCA3-LIKE 1 [OsLFL1], Se5, CO-LIKE 4 [OsCOL4], and Hd16) (Izawa et al., 2000; Peng et al., 2008; Xue et al., 2008; Lee et al., 2010; Hori et al., 2013; Koo et al., 2013). Among the upstream regulators of the \textit{Ehd1–Hd3a/RFT} pathway, OsPRR37 and \textit{DTH8} transcript levels were higher in \textit{oscca1-1} compared with WT under both SDs and LDs, as determined by RT-qPCR (Figure 3, I–L). However, the other upstream regulators Ghd7, OsGI, OsLFL1, Se5, OsCOL4, Hd16, OsMADS51, Ehd2, Ehd3, and Ehd4 showed similar diurnal expression patterns in WT and \textit{oscca1-1} under the same conditions (Supplemental Figure S7). In addition, we measured the transcript levels of OsPRR37, \textit{DTH8}, \textit{Hd3a}, \textit{RFT1}, and \textit{Ehd1} in the fully developed leaves of 56-DAS WT and OsCCA1-OE plants at ZT10 in LDs (Supplemental Figure S8). OsPRR37 and \textit{DTH8} transcript levels were lower in both OsCCA1-OE1 and OE2 lines relative to WT, leading to higher expression levels of \textit{Hd3a}, \textit{RFT1}, and \textit{Ehd1} (Supplemental Figure S8, B–F). Taken together, the results indicate that OsCCA1 represses the expression of OsPRR37 and \textit{DTH8}, followed by the upregulation of \textit{Ehd1}, \textit{Hd3a}, and \textit{RFT1}, leading to promotion of flowering independently of photoperiod.

We determined the epistatic relationship between OsCCA1 and OsPRR37 by measuring OsCCA1 transcript levels in the \textit{osprr37} knockout mutant, which harbors a T-DNA inserted in the 5th exon of OsPRR37, in the leaf blades of WT and \textit{osprr37} at 56 DAS in LDs (Koo et al., 2013). OsPRR37 transcript levels showed a typical diurnal rhythm with higher expression during the day in WT, but not in \textit{osprr37}, as expected (Supplemental Figure S9A). Importantly, the OsCCA1 expression pattern was identical in WT and in the \textit{osprr37} mutant (Supplemental Figure S9B), strongly suggesting that OsPRR37 is epistatic to OsCCA1 in the floral induction pathway.
To investigate whether OsCCA1 affects other circadian clock-associated genes, we determined the diurnal expression patterns of OsPRR1, OsPRR95, EARLY FLOWERING 3-1 (OsELF3-1), and OsPRR73 in leaf blades of WT and oscca1-1 in LDs. The peak times of OsPRR1 and OsPRR95 shifted 3 h early and 3 h later, respectively, in oscca1-1 (Supplemental Figure S10, A and B). However, OsELF3-1 and OsPRR73 transcript levels showed similar diurnal patterns in WT and oscca1-1 (Supplemental Figure S10, C and D).

Next, we monitored the expression of flowering genes in fully developed leaves of WT and oscca1-1 harvested once (SDs) or twice (LDs) a week at ZT1 until heading of WT plants. In LDs, the transcript levels of Hd3a, RFT1, and Ehd1 in oscca1-1 were lower than in WT until 10 weeks after sowing (WAS) and sharply increased up to WT levels thereafter. By contrast, the transcripts of Hd3a, RFT1, and Ehd1 accumulated to lower levels in oscca1-1 throughout development under SDs (Supplemental Figure S11, A–F). The peak times of OsPRR37 (LDs, 8 WAS; SDs, 5 WAS) and DTH8 (LDs, 8 WAS; SDs, 6 WAS) were higher in oscca1-1 than in WT under both daylengths (Supplemental Figure S11, G–J). However, Ghd7, a negative regulator of Ehd1 in LDs, showed similar expression patterns in WT and oscca1-1 (Supplemental Figure S11, K and L). Thus, the late flowering of oscca1-1 is likely due to reduced expression of Ehd1 through de-repression of OsPRR37 and DTH8.

OsCCA1 binds to the promoter regions of OsPRR37 and DTH8

OsCCA1 can specifically bind to target gene promoters containing the G-box motif (CACGTG), CCA1-binding site (CBS, AAA/CAATCT), or evening element (EE, AAAATATCT), or even motif (CBS, AAAATATCT) (Schindler et al., 1992; Wang et al., 1997; Harmer et al., 2000). We therefore investigated whether OsCCA1 binds to the promoter regions of OsPRR37, DTH8, and/or Ehd1, all of which have possible G-box motifs, CBSs, and EEs (Figure 4A). Chromatin immunoprecipitation (ChIP) assays showed that OsCCA1 binds to the promoter regions of OsPRR37 (P3) and DTH8 (P1) with a G-box motif, but not to other OsPRR37, DTH8, or Ehd1 promoter regions (Figure 4B).

We also conducted a yeast one-hybrid (Y1H) assay to determine if OsCCA1 directly binds to the promoter of OsPRR37, DTH8, or Ehd1. OsCCA1 directly bound to the OsPRR37-a promoter region, which includes the G-box motif, but did not bind to other cis-elements of the OsPRR37, DTH8, or Ehd1 promoters (Figure 4C and Supplemental Figure S12). Taken together, OsCCA1 directly binds to the OsPRR37 promoter to regulate OsPRR37 transcription. However, it is possible that OsCCA1-interacting protein(s) indirectly bind to the DTH8 promoter to modulate its expression in an OsCCA1-dependent manner.

The effects of OsCCA1 on agronomic traits

Altering the expression of circadian clock genes affects plant architecture as well as flowering time (Izawa et al., 2011; Zhao et al., 2012; Koo et al., 2013; Yang et al., 2013). We thus evaluated several agronomic traits in WT, oscca1-1, and OsCCA1-OE1 plants grown in paddy fields under NLD conditions (>14-h light/day, 37°N latitude, Suwon, South Korea) and recorded the trait measurements when the plants were harvested in the fall. While plant height was comparable between WT and oscca1-1, OsCCA1-OE1 plants were slightly shorter than WT (Figure 5A). We observed no significant difference for panicle length in WT, oscca1-1, and OsCCA1-OE1 plants (Figure 5B). However, the number of panicles per plant, number of grains per panicle, and 500-grain weight were much lower in oscca1-1 and OsCCA1-OE1 than in WT (Figure 5, C–E), resulting in decreased yield per plant in both oscca1-1 and OsCCA1-OE1 plants compared with WT (Figure 5F). Notably, spikelet fertility was lower in oscca1-1 but higher in OsCCA1-OE1 compared with WT (Figure 5, G and H). Taken together, adequate OsCCA1 expression levels appear to be an important determinant of grain yield under NLD conditions.

Natural OsCCA1 alleles contribute to the extension of rice cultivation regions

Natural variation in heading date-associated genes has allowed rice to adapt to a wide range of latitudes (Koo et al., 2013; Kwon et al., 2014). To examine this variation, we surveyed SNPs in the OsCCA1 coding sequence and the associated flowering time in cultivated rice varieties (O. sativa) from the IRGCIS database (Mansuet et al., 2017). The rice varieties were cultivated at an International Rice Research Institute facility in the Philippines, which has a tropical low-latitude climate and natural SD (NSD) conditions (Kim et al., 2018; Wu et al., 2020). The analysis of SNPs in 2,014 O. sativa accesses identified three nonsynonymous SNPs that can be classified into four haplotypes (OsCCA1a, OsCCA1b, OsCCA1c, and OsCCA1d). Among the three resulting aa differences in cultivated rice, the T418A polymorphism distinguished japonica- and indica-type OsCCA1 alleles (Figure 6A). The OsCCA1a and OsCCA1b haplotypes mainly belonged to temperate and tropical japonica varieties, respectively, while the OsCCA1c and OsCCA1d haplotypes largely consisted of indica varieties such as ind1, ind2, ind3, and indx (Figure 6B; Alexandrov et al., 2015).

Looking at the association between flowering time and each OsCCA1 haplotype, we determined that the OsCCA1 haplotypes carrying japonica-type alleles, OsCCA1a and OsCCA1b, exhibit earlier flowering phenotypes, while rice varieties carrying indica-type alleles (OsCCA1c and OsCCA1d) flowered much later (Figure 6C). The T418A and A653T polymorphisms appeared to delay flowering in oscca1 null mutants compared with WT (Figure 2), we hypothesize that japonica rice cultivars carry functional OsCCA1 alleles that can more strongly induce early flowering than the alleles carried in indica rice cultivars. Plotting the geographic distribution of each OsCCA1 haplotype on the map of Asia revealed that the japonica- and indica-type OsCCA1 alleles...
are widely distributed from high-latitude areas to low-latitude areas (Figure 6D). Taken together, these results indicate that natural variation in OsCCA1 has contributed to the northward expansion of rice cultivars from tropical to temperate climate zones.

OsCCA1 alleles were derived from OrCCA1 alleles of the wild rice Oryza rufipogon during rice domestication

Cultivated rice (O. sativa) was domesticated from wild rice (O. rufipogon) thousands of years ago (Khush, 1997). The phylogenetic analysis of ~5 million SNPs in 446 O. rufipogon accessions classifies the O. rufipogon species into three types (Or-I, Or-II, and Or-III) (Huang et al., 2012). The Or-III type that existed in Southern China is considered to be the ancestral progenitor of O. sativa japonica, whereas O. sativa indica cultivars descended from Or-I in South and Southeast Asia. We surveyed SNPs in the coding sequence of O. rufipogon CCA1 (OrCCA1) from the OryzaGenome database (https://shigen.nig.ac.jp/rice/oryzabase/).

In contrast to the SNPs found in O. sativa, two nonsynonymous SNPs classified 446 O. rufipogon accessions into three haplotypes (OrCCA1a,b, OrCCA1c, and OrCCA1d) (Figure 7A). Since the CCA1 residue F486 is missing in O. rufipogon, the two haplotypes OsCCA1a and OsCCA1b were identical in O. rufipogon and thus designated OrCCA1a,b. Notably, the OsCCA1c and OsCCA1d haplotypes corresponded to the SNPs behind OrCCA1c and OrCCA1d, respectively. The distribution of haplotypes among O. rufipogon rice subspecies showed that 94% of species with the OrCCA1a,b haplotype belong to the Or-III type, while 58% and 71% of Or-I and Or-II types harbored the OrCCA1c and OrCCA1d haplotypes, respectively (Figure 7B). The geographic distributions of OrCCA1 haplotypes in Southern China, Southern Asia, and India indicated that the OrCCA1a,b allele is distributed in Southern China, while wild species carrying OrCCA1c and OrCCA1d existed in Southern Asia and India (Figure 7C).

Discussion

OsCCA1 participates in a unique Ehd1 pathway

A recent study revealed that OsLHY sets the critical day-length (CDL, the length of day below which plants will flower) of rice flowering through the conserved OsGI–Hd1 pathway (Sun et al., 2021). Hd1 promotes flowering under SDs, but represses it under LDs (Zhang et al., 2017). Loss-of-function of OsLHY raises OsGI expression, enhancing the dual function of Hd1 on Ehd1 expression, thereby setting the CDL in rice (Figure 3 and Suplemental Figure S7). OsCCA1 (LOC_Os08g06110, also named OsLHY) is an
ortholog of Arabidopsis CCA1 (AtCCA1; At2g46830) and LHY (AtLHY; At1g01060), encoding core components of the Arabidopsis circadian clock (Alabadí et al., 2002; Mizoguchi et al., 2002; Murakami et al., 2007). The rice genome has one single-copy gene (LOC_08g06110) whose protein is orthologous to both CCA1 and LHY (Zhang et al., 2021); therefore, this protein has been named OsCCA1 and OsLHY in different studies. In this study, we used the name OsCCA1 for the protein encoded by LOC_08g06110 and showed that the OsCCA1–OsPRR37/DTH8–Ehd1 module is a unique pathway controlling the expression of the rice floral integrators Hd3a and RFT1 (Figure 8).

The MYB domain in CCA1 recognizes diverse cis-elements in the promoters of target genes that participate in several regulatory pathways affecting multiple aspects of plant development. For instance, in Arabidopsis, although the EE occurs more often than the CBS, CCA1 binds to both EE and CBS cis-elements to regulate the expression of its downstream targets (Peng and Neff, 2020). Likewise, LHY binds to a G-box within its own promoter to form autoregulatory feedback loops (Spensley et al., 2009; Adams et al., 2015). Furthermore, OsLHY and AtLHY directly bind to the CBS and EE in the OsGI and AtGI promoters in rice and Arabidopsis, respectively (Park et al., 2016). Here, we searched for EE, CBS, and G-box motifs in the promoter regions of OsPRR37, DTH8, or Ehd1 (Figure 4A). Our ChIP and Y1H assays revealed the distinctive regulatory mechanisms of OsCCA1 for rice flowering (Figure 4, B and C). First, OsCCA1 directly binds to the OsPRR37 promoter containing the G-box motif rather than the EE or CBS element that is
bound by AtLHY or OsLHY, respectively. Second, OsCCA1 indirectly binds to a DTH8 promoter fragment that carries a G-box motif. In Arabidopsis, CCA1 physically interacts with the basic leucine zipper (bZIP) TF LONG HYPOCOTYL 5 (HY5) to enhance its binding affinity to the G-box motif in the promoter of CHLOROPHYLL A/B BINDING PROTEIN 1 (CAB1) (Andronis et al., 2008). OsbZIP48 is the rice HY5 ortholog and has pleiotropic effects on light-mediated plant development (Burman et al., 2018). Thus, it is possible that OsCCA1 may recruit regulators including HY5 orthologs, which can directly bind to the DTH8 promoter. Third, OsCCA1 does not bind to the Ehd1 promoter for floral induction. OsPRR37 and DTH8 are LD-dependent floral repressors that contribute to the generation of robust Ehd1 mRNA oscillations (Wei et al., 2010; Koo et al., 2013; Liu et al., 2018; Hu et al., 2021). Furthermore, since OsPRR37 is epistatic to OsCCA1 (Supplemental Figure S9), it is possible that OsCCA1 induces Ehd1 expression by downregulating OsPRR37 or DTH8, although further studies are required to verify the molecular mechanisms of OsCCA1 regulation. In contrast to OsCCA1 functioning as a negative regulator of OsPRR37 in rice, in Arabidopsis, AtCCA1 and AtLHY promote the expression of the OsPRR37 ortholog PRR7 (Farré et al., 2005). In addition, the Arabidopsis PRR family members PRR5, PRR7, and PRR9 repress the expression of AtCCA1/AtLHY through a negative feedback loop (Nakamichi et al., 2010).

Allelic variation at OsCCA1 may improve regional adaptation

The domestication of cultivated rice (O. sativa) has been achieved by selection of natural alleles of floral regulators. For instance, allelic variation of OsPRR37, a suppressor of LD-dependent flowering, contributed to the adaptation of
rice cultivars to a wide range of latitudes (Koo et al., 2013). The *japonica* rice varieties “H143” and “Dongnong416” harbor nonfunctional *ospr37* alleles and flower extremely early, allowing these rice cultivars to be cultivated in northernmost regions around 43–53°N latitude. Interestingly, although other *japonica* rice varieties “Nipponbare (NB),” “DJ,” and “Koshihikari (KS)” carry functional *OsPRR37* alleles, they are mainly cultivated in high-latitude regions (Koo et al., 2013). We speculate that regional adaptation of NB, DJ, and KS is due to the reduced photoperiod sensitivity conferred by the *japonica*-type *OsCCA1* haplotype *OsCCA1a* (Supplemental Figure S13). The repression of *OsPRR37* expression by a strong functional *OsCCA1a* allele would mimic the *OsPRR37* malfunction seen in NB, DJ, and KS.

Similarly, DTH8 is another non-inductive LD-dependent floral regulator that delays rice flowering by suppressing *Ehd1* expression (Wei et al., 2010). The NB cultivar carries a functional *DTH8* allele that should result in late flowering in NLD conditions because of greater photoperiod sensitivity. However, *OsCCA1a* represses the expression of *OsPRR37* and *DTH8* (Figure 3, I–L and Supplemental Figure S8, B and C), leading to early flowering with a weak photoperiod sensitivity in NLD conditions, which allows NB to be cultivated in high latitude regions.

We wondered whether *indica*-type *OsCCA1* alleles (*OsCCA1c* and *OsCCA1d*) can repress *DTH8* expression. Contrary to *DTH8* protein function in LD conditions, *DTH8* promotes flowering in SD conditions by activating the transcription of the floral integrators *Hd3a* and *RFT1* (Yan et al., 2011). The *indica* rice varieties “Kasalath (KA)” and “93-11” harbor an *indica*-type *OsCCA1* allele (Supplemental Figure S13). Considering the strong photoperiod sensitivity of KA and 93-11 with their functional *DTH8* allele (Yan et al., 2011), *indica*-type *OsCCA1* appears to confer either a weak or no repression on *DTH8* expression, thus increasing photoperiod sensitivity and contributing to the adaptation of this *indica* rice variety to low latitude regions.
We speculated that SNPs in OsCCA1 could determine the OsCCA1 haplotypes (Figure 6). The first SNP (T418A) distinguishes between two cultivated rice varieties; *japonica* varieties (temperate, subtropical, and tropical) and *indica* varieties (ind1, ind2, ind3, and indx). The second SNP (F486S) differentiates the *japonica* varieties between OsCCA1a and OsCCA1b. The third SNP (A653T) divides the *indica* varieties into OsCCA1c and OsCCA1d.

Some natural alleles of OsCCA1 were inherited from wild rice (*O. rufipogon*)

Wild rice (*O. rufipogon*), the common ancestor of cultivated rice (*O. sativa*), grows in the tropics (Londo et al., 2006). However, current rice varieties are widely grown from 53°N to 40°S (Fujino et al., 2019). Nonfunctional alleles of flowering suppressors such as *Hd1*, *DTH8*, *Ghd7*, and *OsPRR37*, contributed to this regional adaptation by promoting flowering in non-inductive LD conditions (Xue et al., 2008; Koo et al., 2013; Zheng et al., 2016). Nucleotide polymorphisms in these genes introduce premature stop codons or changes in the open reading frames, resulting in potentially nonfunctional proteins. Therefore, we were interested in the origins of the natural alleles of *OsCCA1* and *DTH8* in wild rice (*O. rufipogon*). Although wild rice has SNPs that introduce aa changes, we did not detect nonfunctional alleles for *OsPRR37* or *DTH8* in *O. rufipogon*. Therefore, we conclude that nucleotide polymorphisms are derived in cultivated rice causing malfunction of *OsPRR37* or *DTH8* are derived from artificial selection during domestication and breeding, not from wild rice.

The selection of OsCCA1 alleles during domestication appears to have differed from the selection that occurred for OsPRR37 and DTH8 alleles. Two SNPs (T418A and A653T) in the *japonica*-type OsCCA1 and *indica*-type OsCCA1 haplotypes are also found in Or-III and Or-I, which are the progenitors of *O. sativa* *japonica* and *O. sativa indica* cultivars, respectively (Figures 6 and 7). For the first SNP (T418A), *japonica* varieties (temperate, subtropical, and tropical) harbor an adenosine (A) and *indica* varieties (ind1, ind2, ind3, and indx) harbor a guanosine (G) (Figure 6B). This SNP is also found in the *O. rufipogon* rice subspecies *Or-III*, which harbors the A allele, and *Or-I*, which harbors the G allele (Figure 7B). The second SNP (F486S) is found in *O. sativa*, but not in *O. rufipogon*; therefore, we cannot exclude the possibility that de novo mutations were selected by natural or artificial processes in the *japonica* varieties. The F486S SNP could be a descendant of other wild rice *Oryza nivara*, another progenitor of *O. sativa* (Vaughan et al., 2008). In conclusion, some natural alleles of OsCCA1 in cultivated rice were inherited from the wild rice *O. rufipogon*.

Possible roles for OsCCA1 in determination of agronomic traits

Previous reports have suggested that OsCCA1 regulates a variety of morphological traits related to yield (Chaudhury et al., 2019; Wang et al., 2020). OsCCA1 integrates sugar responses and the strigolactone signaling pathway, which affects rice tillering and panicle development, as evidenced by the increase in panicle length seen in transgenic NB (*japonica* cultivar) plants overexpressing OsCCA1 (Wang et al., 2020). However, in the current study, we observed no significant difference in panicle length between WT (Dj), oscca1-1, and OsCCA1-OE1 plants (Figure 5). One possible explanation may be due to variety-specific regulatory mechanisms affecting panicle development. In fact, downregulation of OsCCA1 reduces seed length in NB (Wang et al., 2020), but increases the same trait in “Taipei 309” (Chaudhury et al., 2019). Therefore, Dj may have evolved specific regulatory pathways that attenuate the effect of OsCCA1 on panicle development.

OsCCA1 forms a regulatory feedback loop with other circadian clock regulators that control many biological processes involved in growth and development in plants, including flowering time, photosynthesis, starch metabolism, and stress responses (Dodd et al., 2005; Ni et al., 2009; Miller et al., 2015). Rhythmic expression of circadian clock components was considerably affected by photoperiod and temperature (Figure 1, B and C), since NLD conditions of NB cultivation in Nanjing, China (32°N latitude, >13.5 h) (Wang et al., 2020) differ from those of Dj cultivation in Suwon, Korea (37°N latitude, detailed in the “Materials and methods”). Thus, these regional conditions may cause changes in plant morphology such as panicle development between NB and Dj. In addition, since arrhythmic expression of circadian clock genes affects plant architecture (Izawa et al., 2011; Zhao et al., 2012; Yang et al., 2013), the negative effect of oscca1-1 and OsCCA1-OE1 on agronomic traits might be due to the abnormal diurnal expression of OsCCA1 (Figure 5, C–H).

Materials and methods

Plant materials and growth conditions

The rice (*O. sativa*) T-DNA insertional mutant in OsCCA1 (LOC_Os08g06110; PFG_3D-03067; oscca1-1) and its parental *japonica* cultivar “Hwayoung” (WT) were obtained from Crop Biotech Institute at Kyung Hee University, South Korea (Jeon et al., 2000; Jeong et al., 2002; Ryu et al., 2004; An et al., 2005). The WT, oscca1 mutants and OsCCA1-OE1 plants were grown in a paddy field under NLD conditions (>14-h light/day, 37°N latitude, Suwon, South Korea). Unhulled seeds were sown in paddy soil in the greenhouse on May 1 and the seedlings were transplanted to the paddy field on May 22. The daylength during rice cultivation in the paddy field was 14.2 h in May, 14.7 h in June, 14.5 h in July, and 13.6 h in August (calculated at the middle of each month). Rice plants were also grown in growth chambers under LD (14.5 h light/9.5 h dark) or SD (10-h light/14-h dark) conditions. Flowering time (or DTH) was recorded from sowing to emergence of the first panicle in the main culm.
Evaluation of agronomic traits
Plants were grown in a paddy field under NLD conditions (> 14-h light/day, 37°N latitude, Suwon, South Korea). The agronomic traits plant height, panicle length, number of panicles per plant, number of grains per panicle, 500-grain weight, yield per plant, and fertility of WT, oscca1-1, and OsCCA1-OE1 plants were measured after harvest in fall. These experiments were conducted twice with independent samples obtained from different plants.

Plasmid construction and plant transformation
To generate the overexpression construct, the full-length OsCCA1 cDNA was amplified using the primers listed in Supplemental Table S1. The resulting amplicon was subcloned into the pCR8/GW/TOPO TA cloning vector (Invitrogen, Carlsbad, California, USA) and then inserted into the pMDC32 Gateway-compatible binary vector harboring the 35S promoter (Curtis and Grossniklaus, 2003). The pMDC32-OsCCA1 plasmid was introduced into calli generated from the mature embryos of seeds from the japonica rice cultivar “Hwayoung” by Agrobacterium (Agrobacterium strain LBA4404)-mediated transformation (Lee et al., 1999). Agrobacterium-infected calli resistant to 35 mg L⁻¹ hygromycin were transferred to solid Murashige and Skoog (MS) medium containing 2.5 mg L⁻¹ Kinetin and 0.1 mg L⁻¹ NAA, and plantlets were regenerated from calli grown under constant light conditions (90 μmol m⁻² s⁻¹) at 30°C.

To obtain the oscca1-2 mutant allele by CRISPR/Cas9-mediated genome editing, a single guide RNA (sgRNA, 5’-GGAGATTAATTCCTCTGGTG-3’) was designed that targets the OsCCA1 coding region using the CRISPR direct program (http://crispr.dbcls.jp/) (Naito et al., 2015). The custom-designed sgRNA was introduced into the pOs-sgRNA vector, followed by Gateway recombination to mobilize the sgRNA into the destination vector pUbi-Cas9-7 (Miao et al., 2013). The resulting vector was introduced into calli generated from the mature embryos of seeds from the japonica rice cultivar “Dj” by Agrobacterium-mediated transformation (Lee et al., 1999). Transgenic rice calli were selected for hygromycin resistance and confirmed by direct sequencing of genomic PCR products amplified using the target region-specific primers listed in Supplemental Table S1.

RT-qPCR and genomic PCR
To measure the diurnal expression of flowering-time genes, rice leaves were collected every 3 h from 56-DAS plants grown in LDs and 28-DAS plants grown in SDs. To measure the weekly changes in expression of flowering-time genes, rice leaves were detached at ZT1 over the course of plant development in LD and SD conditions. Total RNA was extracted with a MG RNA Extraction Kit (Macrogen, Seoul, South Korea). First-strand cDNA was synthesized with 2 μg of total RNA in a 25-μL volume using M-MLV reverse transcriptase and oligo(dT)₁₅ primer (Promega, Wisconsin, USA), and diluted with 75-μL water. qPCR reactions were performed in 20 μL containing 2 μL of the diluted first-strand cDNA mixture, 10 μL of 2× GoTaq PCR Mix (Promega, Madison, Wisconsin, USA), and 1 μL of 10 pM primer pairs. The qPCR was performed on a LightCycler 2.0 instrument (Roche Diagnostics, Basel, Switzerland) by the relative quantification method using the comparative critical threshold value (Nolan et al., 2006; Caldana et al., 2007; Wong and Medrano, 2005). The qPCR conditions were 95°C for 2 min, followed by 45 cycles at 95°C for 5 s, 59°C for 15 s, and 72°C for 10 s. OsUBQ5 was used as an internal control (Jain et al., 2006). To verify the primer specificity, we used primers that the melting curve showed a single sharp peak. The fragments were sequenced to confirm the target-specific primers by sequencing. Flowering-time genes and their primers used for RT-qPCR analysis are listed in Supplemental Table S1. These experiments were conducted more than three times with independent samples obtained from different tissues.

To identify homozygous oscca1-1 plants, genomic DNA was extracted from an oscca1-1 segregating population using the cetyltrimethylammonium bromide method (Murray and Thompson, 1980). Genomic PCR was performed with the T-DNA plasmid pGA2772 right border primer pGA2772_RB and the OsCCA1 gene-specific primers (Supplemental Table S1). The PCR program included an initial denaturation at 94°C for 3 min, followed by the specified number of cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 40 s, followed by a final extension at 72°C for 5 min.

Subcellular localization
To determine the subcellular localization of OsCCA1, the OsCCA1 coding sequence was cloned into pCR8/GW/TOPO vector (Invitrogen), and then subcloned into pMDC83 vector (Curtis and Grossniklaus, 2003) using Gateway LR clonase II enzyme mix (Invitrogen), resulting in the 35Spro:OsCCA1-GFP plasmid. The control vector (empty pMDC83; 35Spro:GFP) and pMDC83-OsCCA1 plasmids were transiently transfected into onion (Allium cepa) epidermal cells using a DNA Particle Delivery System (Biolistic PDS-1000/He; Bio-Rad, Hercules, USA). The transformed onion epidermal layers were incubated at 25°C on MS plates in darkness for 16 h; green fluorescence was detected using a confocal laser scanning microscope (SP8 X, Leica, Wetzlar, Germany). The GFP fluorescence was detected with 488-nm excitation and 505–530 nm emission wavelengths.

Yeast two-hybrid assays
Yeast two-hybrid assays were performed according to the Yeast Protocols Handbook (Clontech, Kyoto, Japan). To generate GAL4 DNA-BD (binding domain) and GAL4 AD constructs, the OsCCA1 coding sequence was amplified by RT-PCR using gene-specific primers (Supplemental Table S1), and cloned into both pGBK7 and pGADT7 vectors (Clontech). The pairwise BD and AD vectors were co-transformed into the yeast AH109 strain. Positive transformants were then grown on two types of selective media (synthetic defined [SD] –Leu, –Trp or SD –Leu, –Trp, –His)
for 3 days and β-galactosidase activity was determined by liquid assay using chlorophenol red-β-d-galactopyranoside (CPRG; Roche Applied Science, Basel, Switzerland) as substrate according to the Yeast Protocols Handbook (Clontech). These experiments were conducted more than three times with independent clones obtained from different yeast transformants.

Y1H assays

Y1H assays were performed according to the Yeast Protocols Handbook (Clontech). To generate the GAL4 AD construct as prey, the OsCCA1 coding sequence was amplified by RT-PCR using gene-specific primers (Supplemental Table S1) and cloned into the pGADT7 vector (Clontech) for the CPRG assay and the pB42AD vector (Clontech) for the spotting assay, respectively. As a bait, the promoter fragments from possible OsCCA1 target genes (Ehd1, OsPRR37, and DTH8) were amplified by genomic PCR with gene-specific primers (Supplemental Table S1) and the products were then inserted in the pLaCZi vector (Clontech). For the CPRG assay, the yeast YM4271 strain was used as the recipient for bait and prey constructs and β-galactosidase activity was measured by liquid assay using CPRG (Roche Applied Science) according to the Yeast Protocols Handbook (Clontech). For the spotting assay, the EGY48 strain was used as a recipient for bait and prey constructs and β-galactosidase activity was assessed three times with independent clones obtained from different yeast transformants.

Chromatin immunoprecipitation

Rice protoplasts were isolated from leaf sheaths of 10-day-old japonica cultivar “DJ” seedlings grown under LD conditions (14.5-h light/9.5-h dark). The Ubipro:MYC and Ubipro:OsCCA1-MYC constructs (pGA3817 vector; Kim et al., 2009) were transfected into 5 × 106 rice protoplasts by the polyethylene glycol-mediated transfection method (Yoo et al., 2007). Transfected protoplasts were then suspended in protoplast incubation solution (0.5 M mannitol, 20-mM KCl, 4-mM MES, pH 5.8) and kept in darkness for 16 h at room temperature.

For the ChIP assay, the incubated protoplasts were then subjected to crosslinking for 20 min with 1% (w/v) formaldehyde under vacuum at room temperature. The chromatin complexes were isolated and sonicated (Amp 30%, 10-s on/1-min off, five cycles) using a Bioruptor II (Cosmo Bio, Tokyo, Japan) as previously described (Saleh et al., 2008). An anti-Myc polyclonal antibody (Cell Signaling Technology, Danvers, Massachusetts, USA) and protein A agarose beads (Merck Millipore, Burlington, Massachusetts, USA) were used for immunoprecipitation. After reversing the crosslinking and protein digestion, the DNA was purified using a QIAquick PCR Purification kit (Qiagen, Weinbunt, Germany). Finally, qPCR was performed on a LightCycler 2.0 instrument (Roche Diagnostics, Basel, Switzerland) using the following conditions: 95°C for 5 s, 59°C for 15 s, and 72°C for 10 s. OsUBQ5 was used as an internal control. Gene-specific primers are listed in Supplemental Table S1. These experiments were conducted more than three times with the rice protoplasts harvested independently.

Identification of SNPs

To explore the natural variation of OsCCA1, OsPrr37, and DTH8 in cultivated rice (O. sativa) and wild rice (O. rufipogon), we searched for SNPs in the OsCCA1, OsPrr37, and DTH8 coding sequences from the public database; SNPs of O. sativa are from 3K Rice Genome Project from the IRGCIS (Mansueti et al., 2017; https://snp-seek.irri.org/) and SNPs of O. rufipogon are from OryzaGenome Release 2.0 from the National Institute of Genetics under the National Bioresource Project (Huang et al., 2012; https://shigen.nig.ac.jp/oryzabase/). The japonica “NB” allele was used as a reference genome in the SNP analysis. The SNP-associated flowering-time data of cultivated rice varieties were obtained from the IRGCIS.

To confirm the SNPs of OsCCA1, we analyzed the OsCCA1 coding sequences in “DJ,” “KS,” and “Kasalath (KA)” by Sanger sequencing using a cDNA library for each variety. Nucleotide sequences for NB and “93-11” were obtained from the National Center for Biotechnological Information (NCBI) database: NB OsCCA1a (XP_015649844.1); 93-11 OsCCA1d (EEC82927.1).

Construction of the phylogenetic tree

To examine the phylogenetic relationship among CCA1 homologs in land plants, we selected the CCA1 orthologs whose proteins were characterized as functioning in regulating the circadian clock or plant development. The full-length protein sequences were obtained from the NCBI through Basic Local Alignment Search Tool analysis. The phylogenetic tree was constructed using MEGA X software (Kumar et al., 2018) by the maximum-likelihood method with 1,000 bootstrap replication support.

Statistical analyses

The statistical significance of differences between means was determined by conducting two-sided Student’s t tests with P values < 0.05. To determine statistical significance of differences between more than two populations, one-way analysis of variance (ANOVA) with Duncan’s least significant range test and Dunnett’s multiple comparisons tests (P < 0.05) was performed. All central values shown in figures are means of biological or experimental replicates. Box plots were constructed to represent flowering-time distributions (Williamson et al., 1989; Spitzer et al., 2014). In the box plots, the center lines show the medians and plus signs (+) show the mean value; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Statistical analyses were performed using RStudio software (https://www.rstudio.com/) (R version 3.6.1).
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Accession numbers

The gene sequences from this article can be found in the Rice Annotation Project Database or Rice Genome Annotation Project under the following accession numbers: OsCCA1, Os08g06110; OsHd3a, Os06g03630; RFT1, Os06g06300; Hd1, Os06g16370; Ehd1, Os10g32600; OsPRR37, Os07g49460; DTH8, Os08g07740; Ghd7, Os07g15770; OsGl, Os01g08700; OsLFL1, Os01g51610; SE5, Os06g40080; OsCOL4, Os02g39710; Hid16, Os03g57940; OsMADS51, Os01g69850; Ehd2, Os10g28330; Ehd3, Os08g01420; OsPRR1, Os02g40510; OsPRR95, Os09g36220; OsELF3-1, Os06g05060; OsPRR73, Os03g17570.

Supplemental data

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

Supplemental Figure S1. Amino acid sequence alignment of CCA1 proteins.

Supplemental Figure S2. Phylogenetic analysis of CCA1 orthologs in land plants.

Supplemental Figure S3. Subcellular localization and homodimerization of OsCCA1.

Supplemental Figure S4. Identification of the oscca1-1 T-DNA insertional mutant.

Supplemental Figure S5. Flowering time of oscca1-1 and OsCCA1-OEs was measured annually in the field.

Supplemental Figure S6. Phenotypic characterization of the oscca1-2 loss-of-function mutant.

Supplemental Figure S7. Diurnal expression of Ehd1 regulators in the oscca1-1 mutant in LDs.

Supplemental Figure S8. Overexpression of OsCCA1 downregulates OsPRR37 and DTH8 expression and upregulates Ehd1, Hid3a, and RFT1 expression.

Supplemental Figure S9. Diurnal expression of OsCCA1 in the osprr37 mutant.

Supplemental Figure S10. Diurnal expression of circadian clock genes in oscca1-1.

Supplemental Figure S11. Weekly expression of flowering-time genes in oscca1-1.

Supplemental Figure S12. OsCCA1 binds to the OsPRR37-a promoter region.

Supplemental Figure S13. Polymorphic nucleotides at OsCCA1 in five cultivated rice varieties.

Supplemental Table S1. Primers used in this study.

Supplemental Data Set S1. List of the non-synonymous SNPs at OrPRR37 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S2. List of the non-synonymous SNPs at OrDTH8 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S3. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S4. List of the non-synonymous SNPs at OrCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S5. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S6. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S7. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S8. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S9. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S10. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S11. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S12. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S13. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S14. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S15. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S16. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S17. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S18. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S19. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

Supplemental Data Set S20. List of the non-synonymous SNPs at OsCCA1 in 446 O. rufipogon accessions from the National Institute of Genetics under the National Bioresource Project.

Supplemental Data Set S21. List of the non-synonymous SNPs at OsCCA1 in 3,000 rice varieties in the IRGCIS.

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Conflict of interest statement. None declared.

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