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Three CCT domain-containing genes were identified to regulate heading date by candidate gene-based association mapping and transformation in rice

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CCT domain-containing genes generally control flowering in plants. Currently, only six of the 41 CCT family genes have been confirmed to control flowering in rice. To efficiently identify more heading date-related genes from the CCT family, we compared the positions of heading date QTLs and CCT genes and found that 25 CCT family genes were located in the QTL regions. Association mapping showed that a total of 19 CCT family genes were associated with the heading date. Five of the seven associated genes within QTL regions and two of four associated genes outside of the QTL regions were confirmed to regulate heading date by transformation. None of the seven non-associated genes outside of the QTL regions regulates heading date. Obviously, combination of candidate gene-based association mapping with linkage analysis could improve the identification of functional genes. Three novel CCT family genes, including one non-associated (*OsCCT01*) and two associated genes (*OsCCT11* and *OsCCT19*) regulated the heading date. The overexpression of *OsCCT01* delayed flowering through suppressing the expression of *Ehd1*, *Hd3a* and *RFT1* under both long day and short day conditions. Potential functions in regulating heading date of some untested CCT family genes were discussed.

The transition from vegetative to reproductive growth directly determines flowering time in plants. Among monocots, the long-day plants barley and wheat require complete vernalization (a long period with low temperature and long day length) before flowering[1]. In contrast, flowering in rice, a short-day plant, and maize, either a day-neutral or a short-day plant, depends on a short day length[2–4].

The molecular mechanisms underlying flowering have been extensively studied in *Arabidopsis*. It is well known that a circadian clock–controlled flowering pathway comprising the genes *GIGANTEA* (*GI*), *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) promotes flowering under long day conditions (LD)[5–7]. That is, *GI* regulates circadian rhythms and *CO* expression, and *CO* upregulates *FT* expression and results in early flowering under LD[8,9]. This *GI-CO-FT* regulatory pathway is conserved in rice. Under LD, *OsGI* regulates the expression of *Heading date 1 (Hd1)*, the ortholog of *Arabidopsis CO*[4]. *Hd1* negatively regulates *Heading date 3a* (*Hd3a*), the ortholog of *Arabidopsis FT*, and delays the heading date[10]. *Hd1* has a dual function in regulating flowering, as it suppresses flowering under LD but promotes flowering under short-day conditions (SD) by influencing the expression of *Hd3a*[4,11]. A specific flowering pathway mediated by *Ehd1-Hd3a* was also identified in rice[2]. In this rice-specific pathway, *Ehd1* upregulates the expression of *Hd3a* and promotes flowering under LD. *OsGI* is required for induction of *Ehd1* and *Hd3a*. *OsGI-Ehd1* is more dominant for flowering-time control than *OsGI-Hd1* in rice[12,13].

In the photoperiod flowering pathway, *CO* belongs to gene family encoding CCT (CO, CO-LIKE and TOC1) domain proteins. The CCT domain was originally described as a 43 amino acid-long region of homology found in the *Arabidopsis thaliana* proteins CONSTANS (CO), CO-LIKE and TIMING OF CAB1 (TOC1), which have been well characterized as regulators of flowering in *Arabidopsis*[14–16]. CCT family members could be classified into three sub-classes, COL, CMF (CCT MOTIF FAMILY) and PRR (PSEUDORESPONSE REGULATOR) according to their motifs[17]. COLs contain one CCT domain and B-boxes. CMF genes contain only one CCT domain. PRRs share a conserved domain, the receiver-like domain (RLD), along with a CCT domain. The RLD is

similar to the receiver domain of the response regulators in the histidine to aspartic acid (His–Asp) phosphorelay, a versatile signal transduction system[18]. A combined inter-/intra-species comparative and phylogenetic analysis of the CMF, COL and PRR gene families in Poaceae indicates that they evolved prior to the monocot/dicot divergence approximately 200 mya[17].

Several studies have indicated that CCT domain-containing genes are key flowering regulators in rice. *Ghd7*, a CMF class gene, functions upstream of *Ehd1* and *Hd3a* in the photoperiod flowering pathway[19]. *OsCO3*, a COL gene, controls flowering by negatively regulating the expression levels of FT-like genes under SD conditions, resulting in earlier flowering[20]. *OsCOL4*, a constitutive flowering repressor, acts upstream of *Ehd1* and downstream of *OsphyB*[21]. *DTH2*, a COL class gene, promotes heading by inducing *Hd3a* and *RICE FLOWERING LOCUS 1* (*RFT1*) under LD[22]. *Ghd7.1* encodes a PRR37-like protein that represses flowering by suppressing the expression of the downstream genes *Ehd1* and *Hd3a* under LD, greatly enhancing grain productivity[23]. All these studies indicated that the members in each CCT subfamily have potential roles in regulating the heading date. In other cereals, several flowering regulators were identified, including *HvCO1* and *HvCO9*, the homologues of *Hd1 and Ghd7* in barley[24,25]; *Ppd-H1*, *Ppd-D1* and *SbPRR37*, the homologues of *Ghd7.1* in barley, wheat and sorghum, respectively[26–28]; and *ZmCCT*, the homologue of *Ghd7* in maize[3] and *VRN2* in wheat[29]. These genes are all CCT family genes that regulate flowering, which indicates that some genes controlling flowering are conserved at the level of the DNA sequence and share a biological function within cereals, although these cereals have very different growth behaviors and growth regions.

In the last two decades, hundreds of QTLs have been reported for the heading date (http://www.gramene.org/). The major QTLs, such as *Hd1*, *Ghd7* and *Ghd7.1*, have been identified by a map-based cloning strategy[4,19,23]. All these QTLs contain a CCT domain and contribute to rice grain yield through extending the heading date under LD. In rice, it was reported that there are 36 candidate genes in the CCT family, among which 17 genes belong to OsCOL, 14 genes belong to OsCMF, and five genes belong to OsPRR[17]. Although quite a few CCT genes have been characterized for heading date, the functions on flowering of the major members in this family remain unclear.

Association mapping is an efficient alternative to establish the relationships between genes and traits. The major flowering QTL, *Vegetative to generative transition 1*, was associated with flowering and then confirmed by genetic engineering[30]. Based on a diverse panel of Chinese germplasm of rice, several marker loci on chromosome 7 were repeatedly associated with agronomical traits including heading date[31]. In recent years, next-generation sequencing has greatly promoted genome-wide association mapping in plants. Genome-wide association mapping for 14 agronomic traits were conducted based on a population of 373 *indica* lines. Seven genes were associated with heading date[32]. Candidate gene-based association mapping were widely used in maize. For example, one polymorphism in the fifth exon of *ZmGS3* is significantly associated with kernel length in two environments[33]. *ZmCCT*, encoding a CCT domain-containing protein, is associated with the photoperiod response[3]. Gene family is an important resource of candidate gene-based association mapping. The association between *ZmDREB* genes and drought tolerance was evaluated using a diverse population of maize consisting of 368 varieties from tropical and temperate regions[34]. A significant association between the DNA polymorphisms in the promoter region of *ZmDREB2.7* and drought tolerance at seedling stage was identified and further confirmed by transgenic lines in Arabidopsis. But flowering candidate gene-based association mapping was not reported.

In this study, to efficiently identify heading date genes from the CCT family, we first searched for all CCT family members and compared their genomic positions with QTLs for heading date. Then we identified the CCT members with potential functions in regulating heading date via a candidate gene-based association analysis. Finally, we tested the potential functions of 18 candidate CCT members by genetic transformation. Our purpose is to claim how many novel CCT genes regulate rice heading and whether associated CCT genes located in the heading date QTL regions have more chance involved in heading.

Results

Comparison of genome positions between CCT family genes and heading date QTLs. We previously identified a total of 41 CCT family genes in the rice genome[35]. They were mapped to 12 chromosomes according to their physical positions in the genome and included 35 genes but not LOC_Os08g02620, which was reported by Cockram et al.[17] (Fig. 1, Table S2). These genes are unevenly distributed on all 12 chromosomes. Only one CCT family gene is situated on chromosomes 1 and 4, while eight genes are located on chromosome 2. The remaining chromosomes each harbor two to seven CCT family genes. *OsCCT03, OsCCT04*, *OsCCT24* and *OsCCT25*, *OsCCT36* and *OsCCT37*, *OsCCT39* and *OsCCT40* were closely linked to each other, within 300 kb. Accordingly, 133 rice heading date QTLs were collected in the Gramene web site (http://www.gramene.org/, release 37). Finally, a total of 59 heading date QTLs were merged into 12 chromosomes by integrating closely linked QTLs (Fig 1, Table S5). We located 41 CCT family genes and 59 integrated QTLs on 12 chromosomes according to their physical positions by a program compiled by R language (http://www.r-project.org/). Of them, 13 QTLs were isolated, including *OsHY2*, *DTH2*, *Ehd4*, *Hd6*, *Hd16*, *Hd17*, *RFT1*, *Hd3a*, *Hd1*, *Ghd7*, *Ghd7.1*, *Ghd8* (also known as *DTH8* and *Hd5*) and *Ehd1*[2,4,10,19,22,23,36–42]. Chromosome 1 carried the largest number of eight QTLs. On the contrary, chromosome 12 carried the smallest number of three QTLs. A comparison of the genomic positions of the CCT genes with the heading date QTLs showed that 25 CCT family genes were located in the regions of the heading date QTLs. The remaining 16 CCT genes were located outside of heading date QTL regions (Fig 1). Conversely, 39 heading date QTL regions do not contain CCT genes. Among the 13 cloned heading date QTLs, *Qhd14* (*DTH2*), *Qhd31* (*Hd1*), *Qhd36* (*Ghd7*) and *Qhd39* (*Ghd7.1*) are CCT genes, which corresponded to *OsCCT08*, *21*, *26* and *28* in this study. Interestingly, chromosome 1 carried the largest number of heading date QTLs but the smallest number of CCT genes. In contrast, chromosome 2 carried the largest number of heading date QTLs and CCT genes.

Phylogenetic tree of CCT family genes. To understand the evolution of the CCT family genes, protein sequences deduced from Nipponbare were used to construct a neighbor-joining phylogenetic tree based on the conserved CCT domain, the B-box (COL) and the REC domain (PRR). The CCT family was clearly resolved to three subfamilies corresponding to CMF, COL and PRR (Figure 2). These genes included 19 CMF genes, 17 COL genes and five PRR genes (Table S2). *OsCCT26* (*Ghd7*), a CMF subfamily gene, delayed flowering by up to 21 days under LD[19]. *OsCCT28* (*Ghd7.1*), a PRR subfamily gene, delayed flowering by 14 days under LD[23]. *OsCCT08* (*DTH2*), a COL subfamily gene, promoted flowering by eight days under LD[22]. *OsCCT35*, the gene evolutionarily closest to *OsCCT26* (*Ghd7*), is located in the region of *Qhd51* on chromosome 10. *OsCCT11*, the gene evolutionarily closest to *OsCCT28* (*Ghd7.1*), is located in the region of *Qhd16* on chromosome 3. *OsCCT22,* the closest gene to *OsCCT08* (*DTH2*), is located on Chromosome 6.

Expression profiling of CCT family genes. Rice heading is largely regulated by photoperiod. Light is mainly perceived by leaves and

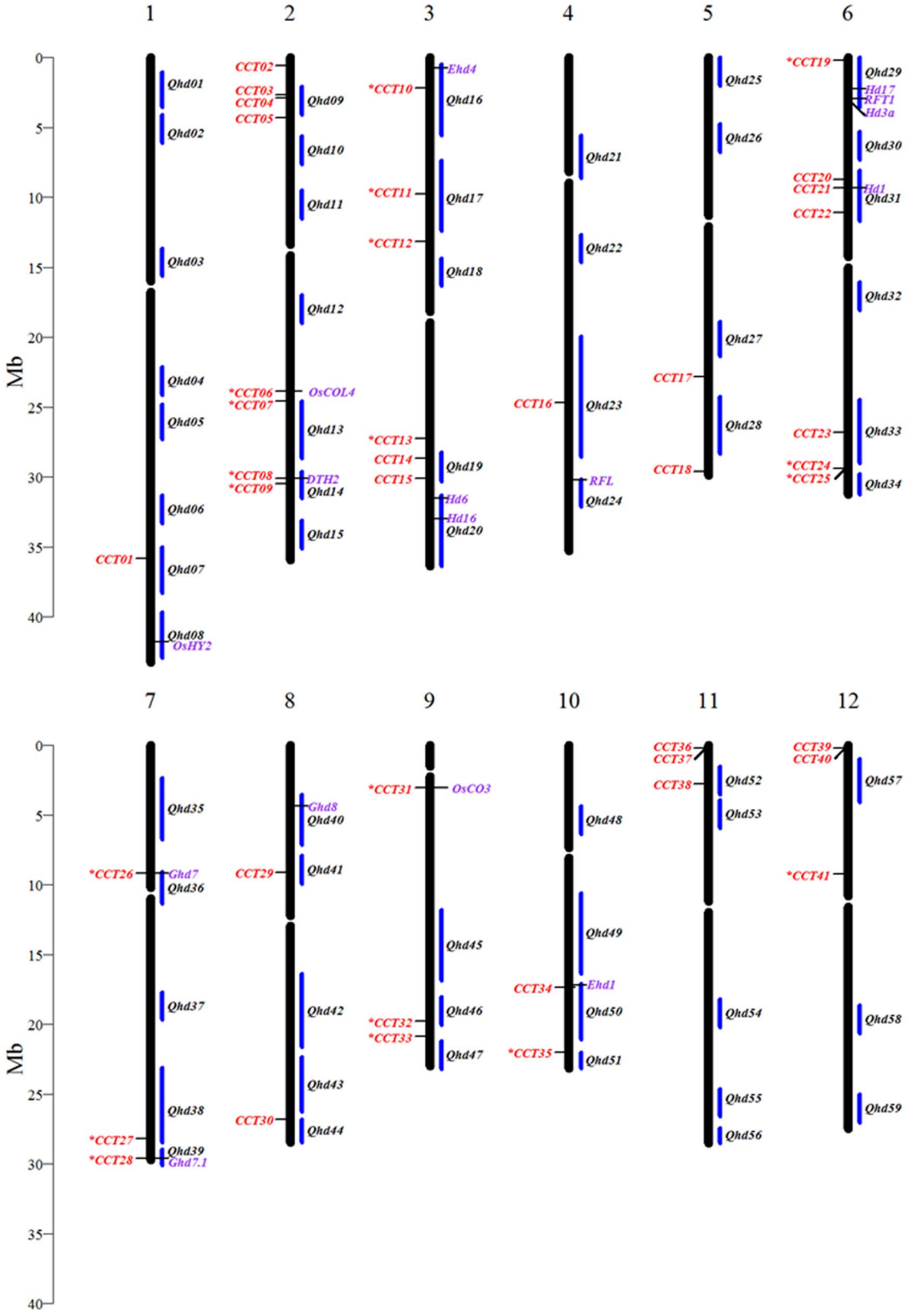

Figure 1 | Positional comparison of heading date QTLs with CCT family genes. CCT genes are indicated on the left side of chromosomes in red; the heading date QTLs are indicated on the right side of the chromosomes in black; and the cloned heading date genes are indicated in purple.

stems before heading. Therefore, we extracted expression data on 35 CCT family genes generated from leaves and stems in the japonica rice Nipponbare (http://ricexpro.dna.affrc.go.jp). In general, the expression levels varied greatly among genes (Figure 2). In the CMF subfamily, four genes (*OsCCT03*, *04*, *13* and *18*) had high expression levels in leaves and stems, two genes (*OsCCT15* and *35*) had considerable expression levels in leaves but were low in stems, seven genes had very low expression levels in all tested tissues, and

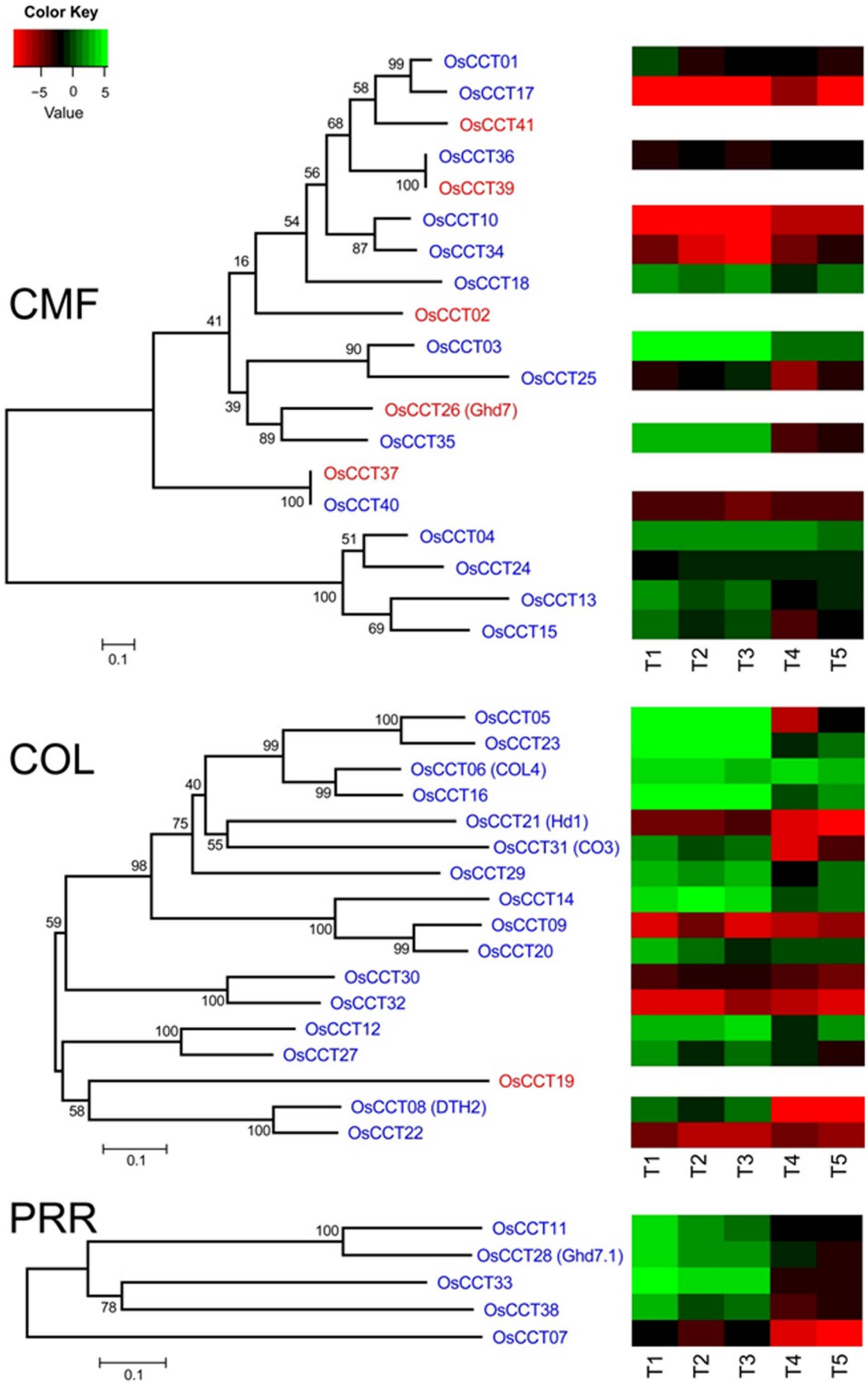

Figure 2 | Phylogenetic tree of rice CCT family genes and their expression profiling in leaves and stems. The phylogenetic tree was constructed based on the amino acid sequences of the proteins. The expression data was extracted from a database (http://ricexpro.dna.affrc.go.jp). The expression data on the 6 genes in red were missing. T1: Leaf blade_27DAT_12:00; T2: Leaf blade_76DAT_12:00; T3: Leaf blade_125DAT_12:00; T4: Stem_83DAT_12:00; T5: Stem_90DAT_12:00.

five genes failed to generate expression data. In the COL subfamily, seven genes, including *COL4,* were highly expressed in all tested tissues, and the other three genes, *DTH2*, *CO3* and *OsCCT05*, were highly expressed only in leaves; four genes, *Hd1*, *OsCCT09, 30* and *32*, had very low expression levels. The PRR subfamily genes, except *OsCCT07*, had higher expression levels in leaves than in stems.

Nucleotide diversity of CCT genes. A total of 2,066 SNPs were identified in 41 CCT family genes from 529 accessions. Of these SNPs, 284 are located in exons, and 662 are located in introns. Other SNPs are located in promoters (943), 5′ UTRs (47) and 3′ UTRs (130). The level of natural variation differed among genes (Table S6). The pairwise nucleotide diversity ranged from one to eight SNPs per kilobase for most CCT genes. The average nucleotide diversity of CCT family genes ($\pi = 2.6 \times 10^{-3}$) is equivalent to that of the whole *Oryza sativa* genome ($\pi = 2.4 \times 10^{-3}$)[43]. *OsCCT18* had the lowest diversity with only one SNP per 10 kilobases in *O. sativa*, and *OsCCT29* had the highest diversity with approximately eight SNPs per kilobase in both *O. sativa* and wild rice. Tajima's *D* of 16 CCT genes, *OsCCT05/08/09/10/13/16/19/21/23/25/28/30/31/35/38/41* ($P < 0.01$), seven CCT genes, *OsCCT01/15/17/20/22/24/26* ($P < 0.001$), reached a significant positive level. The ratio π_c/π_w for *CCT12*, 16 and 18 was less than 0.5, indicating that these genes encountered selection during domestication and genetic improvement (Table S6). In particular, *CCT18* has a very low ratio π_s/π_w of 0.03, indicating a 34-fold reduction in genetic variation in *O. sativa*.

Association mapping for heading date. The 529 accessions with a wide range of heading dates were used to examine associations between CCT family genes and heading date. 529 accessions were classified into five subgroups indica 1 (Ind1), indica 2 (Ind2), an admixture of indica (Ind), tropical japonica (TrJ), temperate japonica (TeJ) and an admixture of japonica (Jap) (Figure S1). No associations were detected between the 41 CCT family genes and heading date in both the whole *indica* group and whole *japonica* group. Then association mapping was conducted within subgroups in case of noise caused by population structure. No associations were detected either in TrJ or Jap subgroups in both years. A total of 19 and 16 CCT family genes were associated with heading date at $P < 0.001$ in 2012 and 2013, respectively. Except *OsCCT10*, *OsCCT11* and *OsCCT35* detected in 2012, associations of the other 16 genes with heading date were detected in both years (Table 1). Among them, five genes are the previously cloned genes, and 10 genes are located in QTL regions. Seven, eight and three CCT family genes were significantly associated with heading date within the subgroups Ind1, Ind2 and Ind in 2012, respectively. In the TeJ subgroup, six and six genes were associated with heading date in 2012 and 2013, respectively. Of them, the associations of *OsCCT07*, *12* and *33* with heading date were only detected in the TeJ subpopulation in 2012. But in 2013, the association of *OsCCT31* with heading date was detected only in TeJ subgroup. The associations of six *OsCCT* genes with heading date were commonly detected in the TeJ subgroup and at least one *indcia* subgroup in 2012 or 2013. The associations of *OsCCT28* were detected in three subgroups in 2012 and four subgroups in 2013. Among all the associated genes, *OsCCT06* showed the highest significant association at $P = 9.4 \times 10^{-7}$ in 2012. In 2013, *OsCCT28* showed the highest significant association at $P = 5.2 \times 10^{-12}$. The heading date-associated genes *OsCCT08*, *OsCCT26* and *OsCCT28* corresponded to the heading date QTLs *DTH2*, *Ghd7* and *Ghd7.1*, respectively, which were identified in bi-parental populations. *OsCCT06* and *OsCCT31* are *CoL4* and *Co3*, respectively, which were reported as repressors of flowering due to the phenotypes of *col4* mutants and *Co3*-overexpressing plants[20,21]. The non-synonymous SNP C/A in the first exon of *CoL4* is associated with heading date. More specifically, the average heading date (111 d) of the C genotype was much later than that (92 d) of the A genotype ($P = 1.3 \times 10^{-7}$). However, the major QTL *Hd1* (*OsCCT21*) was not associated with heading date in any group or subgroup.

Functional validation of CCT family genes by transformation. In total, 41 CCT family genes were classified into four groups according to their associations with heading date and genomic positions. Genes in group 1 were associated with heading date and fell into heading date QTL regions. This group includes 12 genes, including *OsCCT08* and *OsCCT09*. All cloned heading date QTLs/genes belonging to the CCT family except *OsCCT21* (*Hd1*) are classified into group 1. Genes in group 2 fell into heading date QTL regions but were not associated with heading date. There are 14 genes in group 2 such as *OsCCT01*. Genes in group 3 were associated with heading date but not contained in heading date QTL regions. They comprise six genes, including *OsCCT12* and *OsCCT13*. Genes in group 4 were not associated with heading date or contained in heading date QTL regions. There are nine genes in group 4, including *OsCCT02* and *OsCCT22*. To clarify whether genes in different groups have different likelihoods of regulating heading date, we chose a total of 18 genes, including four in group 1, five in group 2, two in group 3 and seven in group 4 to test their functions on heading by transformation (Table 2). We generated transgenic plants overexpressing or silencing each of 18 CCT genes (Figure S2 and Figure S3), among which nine genes were amplified from *japonica* Nipponbare and nine genes were amplified from the late-flowering *indica* cultivar Minghui 63 (Table S4) using gene-specific primers (Table S3). The transgenic plants carrying all nine tested genes in groups 3 and 4 had no altered heading date (Table 2). Among the four tested genes in group 1, *OsCCT11* and *OsCCT19* had effects on heading date. Transgenic plants overexpressing *OsCCT19* (Figure S3) exhibited delayed heading by up to five days, and the *OsCCT11* RNAi plants (Figure S3) delayed heading by up to 12 days under LD (Table 3). The other two tested genes, *OsCCT09* and *OsCCT10*, had no effects on heading. Among the five tested genes in group 2, overexpression of *OsCCT01* (Figure S3) greatly delayed heading by more than 25 days but resulted in a decreased plant height and short panicles (Fig 3). The other four genes had no effects on heading when overexpressed.

Expression pattern of *OsCCT01* and the subcellular localization of OsCCT01. We investigated the diurnal expression pattern of *OsCCT01* in young leaves of Zhonghua 11 using qRT-PCR (Fig S4). The *OsCCT01* transcript was more abundant during the day than in darkness. The expression of *OsCCT01* was low under SD, while its expression peaked at similar levels in the morning under both SD and LD. To assay the sub-cellular localization of *OsCCT01*, the coding sequence of *OsCCT01* was fused to that of yellow fluorescent protein (YFP), and *Ghd7*, a nuclear protein, was fused to cyan fluorescent protein (CFP). The constitutive 35S cauliflower mosaic virus promoter was used to drive expression of the two fused fluorescent proteins. The constructs were co-transfected into rice protoplasts derived from etiolated seedlings. The merged picture showed that OsCCT01 co-localized with GHD7, indicating that OsCCT01 is a nuclear protein (Figure S5).

Photoperiod sensitivity of *OsCCT01*. To investigate the function of *OsCCT01* in the photoperiodic control of flowering, the T_2 generation of transgenic plants was used to examine flowering time under LD and SD (10 h light and 14 h darkness) in a natural field. *OsCCT01*-overexpressing plants exhibited 14 day-later flowering under SD and 25 day-later flowering under LD relative to the negative control plants (Table 4). These results suggest that *OsCCT01* regulates flowering time in rice under both SD and LD. We compared the expression of *OsCCT01* and other key flowering regulators in young leaves between wild-type Zhonghua 11 and *OsCCT01*-overexpressing plants using qRT-PCR. The expression levels of *Ehd1*, *Hd3a,* and *RFT1* were downregulated in the

Table 1 | The 19 CCT family genes associated with heading date using general mixed model in 2012 and 2013

	P (2012)				*P* (2013)			
CCT-ID	Ind	Ind I	Ind II	TeJ	Ind	Ind I	Ind II	TeJ
OsCCT06 (*OsCOL4*)	9.4×10^{-7}				1.5×10^{-8}			
OsCCT07				2.2×10^{-4}	5.2×10^{-5}		2.8×10^{-6}	1.1×10^{-4}
OsCCT08 (*DTH2*)	8.7×10^{-5}	8.1×10^{-4}			1.2×10^{-6}			
OsCCT09		6.4×10^{-6}				1.7×10^{-5}		
OsCCT10		9.0×10^{-4}						
OsCCT11			1.4×10^{-5}					
OsCCT12				2.9×10^{-4}	2.4×10^{-4}			2.4×10^{-4}
OsCCT13		1.2×10^{-4}		4.7×10^{-5}		8.0×10^{-4}		3.1×10^{-6}
OsCCT19	2.7×10^{-4}				1.8×10^{-4}			
OsCCT24	1.3×10^{-4}				6.1×10^{-4}			
OsCCT25	1.8×10^{-4}				8.0×10^{-4}			
OsCCT26 (*Ghd7*)		1.4×10^{-5}		1.4×10^{-5}	8.6×10^{-6}			
OsCCT27	9.1×10^{-4}				5.7×10^{-4}			
OsCCT28 (*Ghd7.1*)	1.4×10^{-4}		1.4×10^{-4}	2.5×10^{-4}	5.2×10^{-12}	6.1×10^{-4}	7.3×10^{-6}	1.3×10^{-4}
OsCCT31 (*OsCO3*)		8.1×10^{-4}						5.6×10^{-4}
OsCCT32			3.8×10^{-4}				2.8×10^{-5}	
OsCCT33				6.5×10^{-4}	4.7×10^{-6}			1.8×10^{-4}
OsCCT35		4.3×10^{-4}						
OsCCT41		2.3×10^{-4}				2.5×10^{-4}		

transgenic lines compared to the wild-type plants under both SD and LD. In contrast, the transcript levels of *Hd1* were not obviously affected (Figure 4). The expression of other flowering regulators such as *OsGI*, *RID1/OsID1/Ehd2*, *Ehd3*, *Ehd4*, *OsMADS50*, *OsMADS51*, *OsMADS56*, *Ghd7*, *Ghd8/DTH8*, *Ghd7.1* and *DTH2*, was also not affected by *OsCCT01* (Figure S6 and S7). Thus, we deduced that *OsCCT01* acted as a negative regulator upstream of *Ehd1* in the photoperiod flowering pathway under SD and LD.

Discussion

The extent of linkage disequilibrium determines the resolution of association mapping and the number of markers required for genome-wide association analysis[44,45]. Candidate gene-based association analysis has the potential to identify putative functional genes in several species including humans, animals and maize, in which quick linkage disequilibrium decay has been reported[46–48]. Candidate genes frequently come from some gene families, which have been characterized as associating with target traits, from genes involving targeted biosynthesis pathways, such as the tocopherol biosynthesis genes[49], and from the potential genes in small genome regions delimited by map-based cloning[40].

Many crucial flowering-related rice genes contain the CCT domain, such as *Hd1*, *Ghd7*, *Ghd7.1* and *DTH2*[4,19,22,23]. Naturally, the other CCT family genes were regarded as candidate genes for association analysis. A total of 19 CCT genes, including five previously cloned flowering-related genes, were associated with heading

Table 2 | Function of 18 CCT family genes on heading date was tested by transformation

CCT-IDs	*Qhd* regions	Association	Group	Effects on heading date
OsCCT08 (*DTH2*)	*Qhd14*	Yes	one	Yes (Wu et al., 2013)
OsCCT09	*Qhd14*	Yes	one	No (OX)
OsCCT10	*Qhd16*	Yes	one	No (OX)
OsCCT11	*Qhd17*	Yes	one	Yes (RNAi)
OsCCT19	*Qhd29*	Yes	one	Yes (OX)
OsCCT26 (*Ghd7*)	*Qhd36*	Yes	one	Yes (Xue et al., 2008)
OsCCT28 (*Ghd7.1*)	*Qhd39*	Yes	one	Yes (Yan et al., 2013)
OsCCT01	*Qhd07*	No	two	Yes (OX)
OsCCT03	*Qhd09*	No	two	No (OX/RNAi)
OsCCT07	*Qhd13*	No	two	No (OX/RNAi)
OsCCT21 (*Hd1*)	*Qhd31*	No	two	Yes (Yano et al., 2001)
OsCCT29	*Qhd37*	No	two	No (RNAi)
OsCCT38	*Qhd52*	No	two	No (RNAi)
OsCCT06 (*OsCOL4*)		Yes	three	Yes (Kim et al., 2008)
OsCCT31 (*OsCO3*)		Yes	three	Yes (Lee et al., 2010)
OsCCT33		Yes	three	No (RNAi)
OsCCT41		Yes	three	No (RNAi)
OsCCT05		No	four	No (RNAi)
OsCCT17		No	four	No (RNAi)
OsCCT18		No	four	No (OX)
OsCCT22		No	four	No (OX/RNAi)
OsCCT37		No	four	No (OX)
OsCCT39		No	four	No (OX/RNAi)
OsCCT40		No	four	No (OX)

Six heading date genes previously identified were also listed.
The expression levels of targeted CCT genes in transgenic plants please see Figure S2.

Table 3 | Comparison of heading date between positive and negative transgenic plants of three CCT family genes under long day length conditions

		Heading date (d)		
Identity	Expression pattern	Positive T_1 plants	Negative T_1 plants	*P*
OsCCT11	RNAi (ZH11)	86.9 ± 2.8	74.6 ± 4.2	5.8×10^{-5}
OsCCT19	OX (HJ19)	64.2 ± 1.2	59.3 ± 2.0	2.4×10^{-13}
OsCCT01	OX (ZH11)	104.3 ± 2.6	78.3 ± 1.2	2.4×10^{-8}

RNAi (ZH11), RNA interference in the genotype of Zhonghua 11. OX (HJ19) and OX (ZH11) overexpressed in the genotype of Hejiang 19 and zhonghua 11, respectively. Heading date was collected from two replicates.

date. Of these, six were analyzed for functions by transformation. The regulation of heading date by *OsCCT11* and *OsCCT19* was further confirmed by RNAi and overexpression, respectively. Both genes are located in QTL regions. The other four genes (*OsCCT09*, *OsCCT10*, *OsCCT33* and *OsCCT41*) have no effects on heading date. We tested 12 CCT genes not associated with heading date by transformation. Overexpression of one gene (*OsCCT01*) significantly delayed heading. Moreover, an analysis of the expression levels of several key flowering-related genes showed that *OsCCT01* suppressed *Ehd1* and further suppressed *Hd3a* under either SD or LD, resulting in delayed heading. Therefore, OsCCT01 is a flowering suppressor. The effects of *OsCCT01* on plant height and grain yield are distinct from the cloned CCT family genes such as *Ghd7* and *Ghd7.1*. Both *Ghd7* and *Ghd7.1* increase plant height and grain yield[19,23], whereas *OsCCT01* decreases plant height and grain yield mainly by decreasing the length of stem internodes, the number of spikelets per panicle and seed setting rate (Table S8).

There are nine CCT family genes that regulate heading date if including the previously cloned six genes. Among the nine functional genes, five genes were located within QTL regions, and two genes were located outside of QTL regions and were associated with heading date; two genes located in the QTL regions were not associated with heading date. In summary, five of seven associated genes in the QTL regions have effects on heading date, two of six non-associated genes in the QTL regions regulate heading date, two of four associated genes outside of QTL regions regulate heading date and none of the seven non-associated genes situated outside of the QTL regions regulates heading date. Therefore, the combination of association mapping with linkage analysis improved the identification of functional genes.

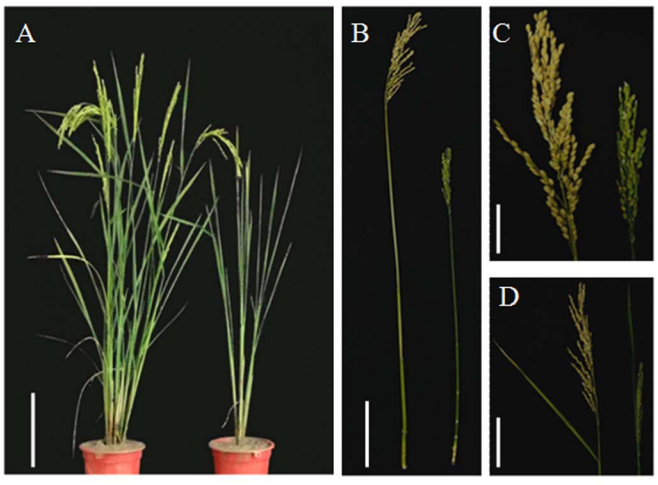

Figure 3 | The phenotypes of plant status (A), main stem (B), panicle (C) and flag leaf (D) in the *OsCCT1* overexpression positive (right) and negative T1 plants (left).

Seven of the nine functional genes, all except *Hd1* and *OsCCT01*, were detected by association mapping. Thus, candidate gene-based association analysis has considerable power in QTL mapping. However, four of the 11 associated genes have no effect on heading date as confirmed by either gene overexpression or gene silencing, and two (*OsCCT21/Hd1* and *OsCCT01*) functional genes were not associated with heading date. That result indicates that type I error and type II error occurred. Type I error is frequently caused by low threshold values. Moreover, low frequency alleles were excluded when conducting association mapping. Thus, it is hard to avoid type II error. In fact, only approximately one half of the heading date-related genes were found to be associated with heading date even using a large number of landraces[43]. In this study, the most possible reason for type I error is a large degree of linkage disequilibrium in the target regions[43] and a close linkage between tested genes and a single heading date gene in the linkage disequilibrium regions. For example, *OsCCT09* and *OsCCT10* are closely linked to *DTH2* and *EHD4*. In addition, it is also possible that functional redundancy exists among CCT genes[50]. Silencing some genes might not cause any phenotypic change. As for *OsCCT01* and *OsCCT19*, changes in heading date were observed in the overexpression plants, but no change was observed in the RNAi plants. Thus, the functions of these CCT family genes (*OsCCT09*, *10*, *29* and *38*) tested by RNAi are uncertain. Knockout mutations created by the high efficiency gene-editing method Clustered Regularly Interspaced Short Palindromic Repeat (CRISPRS)/CRISPR-associated (Cas) systems[51] would be much better than knockdown mutations for testing the functions of family genes.

A negative Tajima's D signifies an excess of low frequency polymorphisms relative to expectations, indicating a selective sweep or purifying selection. A positive Tajima's D signifies balancing selection. The Tajima' D test ($P < 0.01$) indicated that no significant Tajima's D was detected in wild rice. However, in cultivar, Tajima's D values for *OsCCT18* were negative and significant. Clearly, *OsCCT18* was selected during its natural evolutionary process. However, we did not observe any function of *OsCCT18* in the overexpression plants. Therefore, we examined the DNA polymorphisms in eight noncoding regions (ranging from 313 bp to 2,055 bp) flanking *OsCCT18* (Table S4) because noncoding regions eliminate the interference of other genes' variation. We observed a selective sweep with a notable reduction in relative diversity in a small segment (< 50 kb) (Table S7). These results indicated that a

Table 4 | Heading dates of the *OsCCT01* overexpressed T_2 plants

	LD (14 h)		SD (10 h)	
Genotypes	No	HD (d)	No	HD (d)
Positive	**7**	104.3 ± 2.6	**7**	81.6 ± 1.8
Negative	**7**	78.3 ± 1.2	**7**	68.4 ± 2.6
P		2.4×10^{-8}		2.9×10^{-7}

LD long day-length condition; SD short day-length condition; HD heading date; No number of plants investigated.

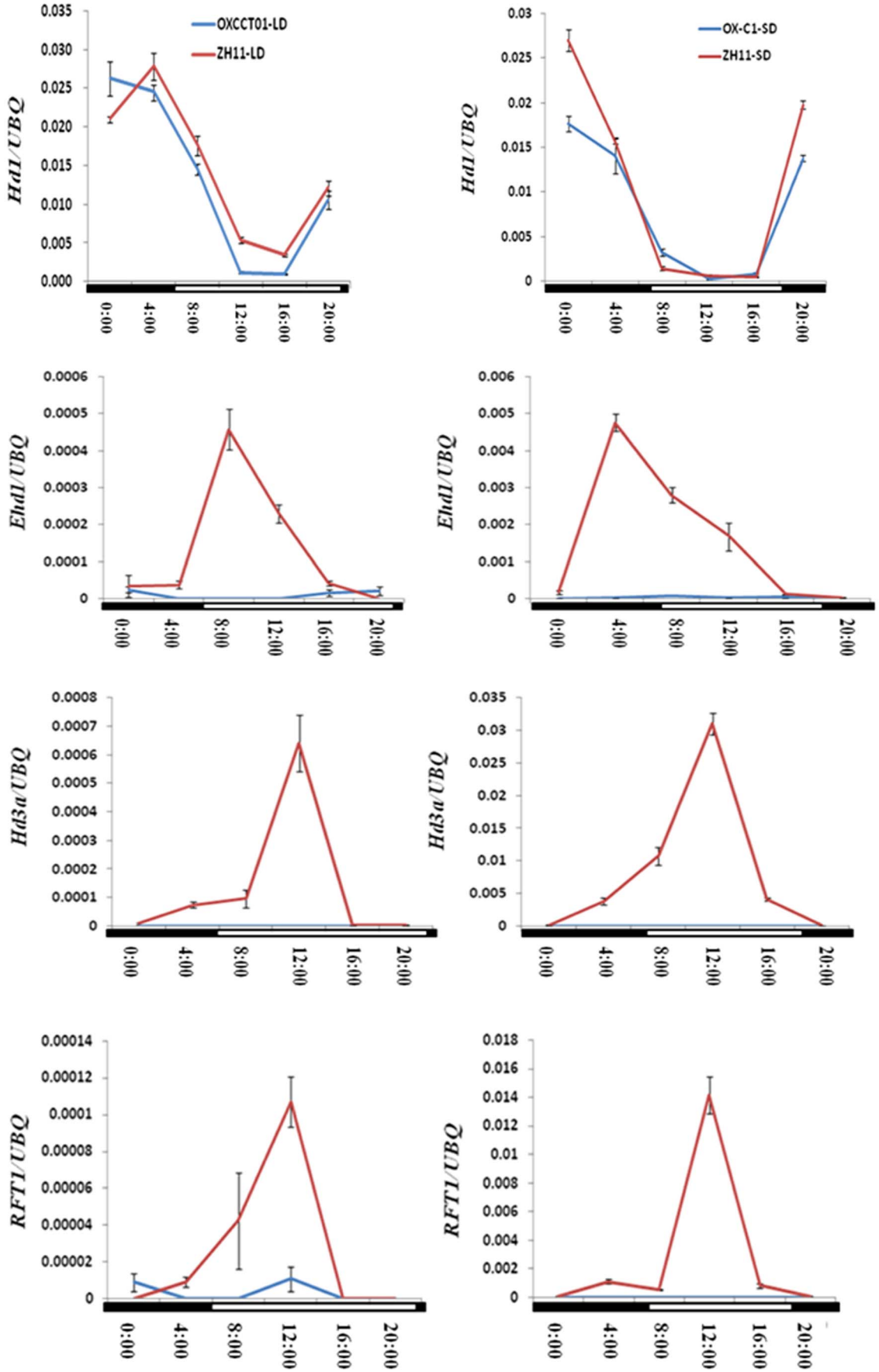

Figure 4 | Diurnal expression patterns of *Hd1*, *Ehd1*, *Hd3a* and *RFT1* in *OsCCT01*-positive transgenic plants and wild-type Zhonghua 11 plants grown under long day-length conditions. X axis indicated the time points for sampling. Y axis indicated the relative expression levels of investigated genes to *UBQ*.

strong selection pressure was imposed on the region surrounding *OsCCT18* during rice domestication. Therefore, *OsCCT18* has no obvious phenotypic function but infrequent selection. Tajima's D values for *OsCCT01*, *17*, *20* and *24* were positive and significant. Huang et al.[43] reported that more than 60 genome regions experienced infrequent selection in cultivars compared to wild rice. Of them, the selected regions (19.9–21.1 Mb in indica/22.8–22.9 Mb in japonica and 29.7–29.9 Mb) contained *OsCCT17* and *18*. It is likely that directional selection on favorable alleles in the regions caused genetic hitchhiking effects on linked *OsCCT17* and *18*.

In this study, in addition to the previously cloned six CCT family genes, three CCT family genes were identified as regulating heading date. We believe that more genes regulate heading date because the functions of the other eight genes associated with heading date remain to be confirmed by transformation. In addition, the functions of two CCT family genes (*OsCCT33* and *41*), which were associated with heading date, were likely misevaluated by RNAi because of gene redundancy. A combination of overexpression and gene knockout methods to test gene function would achieve more reliable results. Currently, it is not clear whether *OsCCT20* and *OsCCT24* regulate heading date. The Tajima test indicated that both genes underwent balancing selection, but the regions surrounding both genes were not reported to suffer selection. Therefore, genetic hitchhiking effects could not be used to explain their infrequent selection; most likely, they are heading date-related genes. *OsCCT11* is the member closest to the major heading date-related gene *OsCCT28* (*Ghd7.1*), and *OsCCT21* (*Hd1*) is close to *OsCCT31 (CO3)*. Both genes regulate heading date. *OsCCT35* and *OsCCT16* are the members closest to the key heading date genes *OsCCT26* (*Ghd7*) and *OsCCT06* (*COL4*), respectively. *OsCCT35* and *OsCCT16* have potential functions in flowering. These CCT family genes should be tested for their functions.

Methods

Measurement of heading date. The Chinese core collection consisting of 202 cultivars and the world core collection of 327 cultivars and an additional 107 common wild rice accessions (*Oryza rufipogon*) were sowed at the farm of Huazhong Agricultural University Wuhan, China on May 19 2012 and on April 17 2013. The basic information for 529 cultivars is available on the RiceVarMap[52] (http://ricevarmap.ncpgr.cn/), and the details of the 107 wild rice accessions are included in Table S1. Seven 25-day-old seedlings from each accession were transplanted in a single row with a distance of 16.5 cm between plants and 26.4 cm. Field management was performed according to normal agricultural practices. The heading date was defined as the number of days from sowing to the appearance of the first panicle. The 5 plants in the middle were used to score the heading date. The average heading date of the 5 plants was used for data analysis.

Integration of heading date QTLs. The key words "heading date in rice" and "flowering in rice" were used as queries to search against the annotation database of the rice genome (http://www.gramene.org/, release 37). Then, the physical positions of all the collected QTLs were obtained through aligning the sequences of each QTL's flanking RFLP marker to the rice reference genome (Gramene Annotated Nipponbare Sequence 2009) by BLAST (BLAST-like alignment search tool)[53] or performing an e-PCR[54] using the pair of primers of corresponding SSR markers to the whole genome sequence[55]. Considering a 1-LOD confidence interval of more than 5 cM for a QTL in a primary mapping population, each couple of QTLs with a physical distance less than 1250 kb (approximately 5 cM) were merged into one.

Identification of CCT family genes in rice. The conserved CCT domain sequence EREAKLMRYKEKRKKRCYEKQIRYASRKAYAEMRPRVRGRFAKE of the known GHD7 protein was used to search (using the tBLASTn and BLASTp programs) against the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/)/ NCBI (http://www.ncbi.nlm.nih.gov)/DRTF (http://drtf.cbi.pku.edu.cn/index.php)[35]. A total of 41 CCT genes were identified and named sequentially according to their genome positions (Table S2). The unrooted phylogenetic tree of CCT family members was constructed with the MEGA5.2.2 program by the neighbor-joining method and bootstrap analysis (1000 replicates)[56]. The expression pattern heat map of the CCT family members was drawn by the R language, and expression profiling of 35 members came from RiceXPro (http://ricexpro.dna.affrc.go.jp).

Nucleotide diversity analysis. The genome sequences of the 529 accessions were downloaded from RiceVarMap (http://ricevarmap.ncpgr.cn/). The sequence of each CCT family gene was extracted from the dataset. Each sequence included the gene body, approximately 2 kb of the promoter region and approximately 500 bp of the 3′ flanking region. The average number of nucleotide differences per site between two random sequences (π), the Watterson estimator (θ_w), nucleotide diversity and Tajima's D statistics were calculated using the DnaSP 5.0 program[57]. The value of π_c/π_w was calculated with the average proportion of pairwise difference per base pair in cultivated rice (π_c) divided by that in wild rice (π_w).

Candidate gene-based association mapping. The population structure of 529 accessions was considered when conducting the association analysis. The parameter of the number of ancient clusters K was set from two to seven to obtain different inferences. The highest likelihood for a subpopulation was obtained with $K = 6$[52] (http://ricevarmap.ncpgr.cn). Associations between SNPs in CCT genes and heading date were analyzed with a general linear model (GLM) using the software TASSEL[58]. The Bonferroni-adjusted significance threshold is set as 0.05/n (n polymorphism)[59]. For most candidate genes, n is fluctuated from 40 to 60 with an average of approximate 50 within each subgroup. Hence $P<0.001$ was set the threshold for claiming association. Analyses were conducted with population structure estimates, using the *Q-matrix* obtained from the RiceVarMap (http://ricevarmap.ncpgr.cn).

Complementation test of heading date-related CCT members. Genome DNA fragments or coding DNA sequences of 18 CCT genes were amplified from Minghui 63 or Nipponbare DNA with gene-specific primers (Table S3) using LA Taq polymerase (Takara) with high fidelity. The transformation information for 18 CCT genes is listed in Table S4. The amplicons without mutations were identified by sequencing and inserted into the binary vectors pCAMBIAI1301s and ds1301. The resulting constructs were introduced into Zhonghua 11 or Hejing 19 by *Agrobacterium tumefaciens*-mediated transformation using immature embryos as subjects[60]. T_1 and T_2 families were sown in the field bed on 16 May, and 30 plants of T_1 families for each candidate gene were transplanted on 15 June 2011 and 2012 in the Experimental Station of Huazhong Agricultural University, Wuhan, China. The wild-type accessions Zhonghua 11 and Hejiang 19 were grown as the controls. Seven *OsCCT01* overexpressed plants and seven negative plants were treated in the short day (10 h day length) and long day (14 h day length) conditions till heading respectively.

RNA extraction and expression analysis. The seeds of negative and positive *OsCCT01* transgenic plants and wild-type were sown in pots in a greenhouse. After 32 d of growing under approximately neutral day-length conditions (12 h of light per day), half of the plants were moved to a short day condition (10 h/d) in a phytotron, and the other half were moved to a long-day condition (14 h/d) in a different phytotron. The light intensity in the phytotrons was set at 10,000 lx, with the temperature set to 30°C in the light period and 26°C in the dark period. After an additional seven days of growth, young leaves were simultaneously harvested from three different plants as three biological repeats for each treatment and stored in liquid nitrogen. To test their circadian expression patterns, the samples were collected once every four hours for 24 hours. In addition, young fresh leaves from other CCT genes' transgenic plants were separately sampled for RNA extraction at seedling stage. 10 day seedling and 4 cm young panicle were sampled from negative and positive *OsCCT01* transgenic plants for RNA extraction to detected rice yield genes and plant height involved genes, such as GAs.

Total RNA was extracted from the leaves using an RNA extraction kit (TRIzol reagent, Invitrogen) for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Approximately 3 μg total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen) in a volume of 150 μl to obtain cDNA. We investigated the transcription levels of 18 CCT genes in their corresponding transgenic plants and photoperiod genes including *Hd1*, *Ehd1*, *Hd3a* and *RFT1* in *OsCCT01* transgenic plants. *Ubiquitin* was set as the internal control (LOC_Os03g13170). All the primers used for qRT-PCR are presented in Table S3. qRT-PCR was run in a total volume of 15 μl containing 3.6 μl of the reverse-transcribed product described above, 0.25 μM gene-specific primers and 7.8 μl FastStart Universal SYBR Green Master (Rox) superMIX (Roche, Mannheim, Germany) on an Applied Biosystems ViiA 7 Real-Time PCR system according to the manufacturer's instructions. The measurements were obtained using the relative quantification method.

Expression analysis and sub-cellular localization. The coding sequence of *OsCCT01* (MH63) was fused to PM999-YFP. The fusion protein with insertion in the correct direction was co-transfected into rice protoplasts with *Ghd7:CFP* as described by Zhou[61], with minor modifications. The fluorescence image was obtained using a confocal microscope (Leica, Germany) after incubating the transformed cells in the dark at 28°C for 20 h.

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Author contributions

Conceived and designed the experiments: Y.X. Performed the experiments: L.Z., Q.L., H.D., Q.H., L.L. and G.L. Analyzed the data: L.Z., C.T., W.Y., H.Z. and W.X. Wrote the paper: Y.X. and L.Z.

Additional information

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