RESEARCH PAPER

Fine-tuning of the setting of critical day length by two casein kinases in rice photoperiodic flowering

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Abstract

Many short-day plants have a critical day length that fixes the schedule for flowering time, limiting the range of natural growth habitats (or growth and cultivation areas). Thus, fine-tuning of the critical day-length setting in photoperiodic flowering determines ecological niches within latitudinal clines; however, little is known about the molecular mechanisms controlling the fine-tuning of the critical day-length setting in plants. Previously, we determined that florigen genes are regulated by day length, and identified several key genes involved in setting the critical day length in rice. Using a set of chromosomal segment substitution lines with the genetic background of an elite temperate japonica cultivar, we performed a series of expression analyses of flowering-time genes to identify those responsible for setting the critical day-length in rice. Here, we identified two casein kinase genes, Hd16 and Hd6, which modulate the expression of florigen genes within certain restricted ranges of photoperiod, thereby fine-tuning the critical day length. In addition, we determined that Hd16 functions as an enhancer of the bifunctional action of Hd1 (the Arabidopsis CONSTANS ortholog) in rice. Utilization of the natural variation in Hd16 and Hd6 was only found among temperate japonica cultivars adapted to northern areas. Therefore, this fine-tuning of the setting of the critical day length may contribute to the potential northward expansion of rice cultivation areas.

Keywords: Casein kinase, critical day-length, photoperiodic flowering, rice, short-day plants.

Introduction

Floral transition, the major developmental switch from the vegetative to reproductive phase in plants, is regulated by both endogenous and environmental signals. Photoperiodic flowering, one of the most important biological systems in controlling floral transition, is regulated by light signals and the plant’s endogenous circadian rhythm (Thomas and Vince-Prue, 1997). Rice photoperiodic flowering has been investigated extensively as a model system of short-day (SD) plants. The rice florigen gene Heading date 3a (Hd3a) is regulated according to the recognition of critical day length, and several key genes have been identified that are necessary for setting this day length (Itoh et al., 2010).

The critical day length determines whether a plant will flower under certain cultivation environments. For example,
several rice cultivars usually grown in tropical areas fail to flower in temperate areas, as it becomes too cold to differentiate the panicles at the apex when day length falls below the critical level. Thus, the genetic setting of critical day length may be a major factor determining the range of cultivation for rice cultivars. Natural habitats of various Japanese duckweed (Lemma) accessions are distributed latitudinally, showing associations with their critical day length (Yukawa and Takimoto, 1976; Beppu and Takimoto, 1981). However, the molecular genetics underlying the fine-tuning of critical day length has yet to be elucidated. In rice, $\text{Hd3a}$ gene expression was found to be affected by a single long-day (LD) stimulus, and the resulting change in $\text{Hd3a}$ produced late flowering (Ishikawa et al., 2011). Two distinct circadian-clock gates controlling the $\text{Ehd1}$ floral activator and the $\text{Ghd7}$ floral repressor sensitively and accurately regulate the threshold of $\text{Hd3a}$ expression (Itoh et al., 2010). Recently, we reported that the formation of a transcriptional complex containing both $\text{Hd1}$ and $\text{Ghd7}$ proteins could repress $\text{Ehd1}$ expression under LD conditions (Nemoto et al., 2016).

In rice, many quantitative trait loci (QTLs) have been implicated in regulating flowering time under field conditions, and positional cloning has been used to isolate the corresponding genes or functional nucleotide polymorphisms (Matsubara et al., 2014). QTL analyses for flowering-time (or heading date) conducted on two temperate $\text{japonica}$ cultivars, ‘Nipponbare’ and ‘Koshihikari’, revealed the involvement of two alleles, $\text{Hd16}$ and $\text{Hd17}$ (Matsubara et al., 2008), and the causative genes were also identified (Matsubara et al., 2014). $\text{Hd16}$ and $\text{Hd17}$ encode a casein kinase I (CKI) and an ortholog of Arabidopsis $\text{EARLY FLOWERING 3}$ (ELF3), respectively (Matsubara et al., 2012; Hori et al., 2013). The Koshihikari allele of $\text{Hd16}$ promotes flowering under natural conditions and decreases the kinase activity in comparison with the Nipponbare allele (Hori et al., 2013). Because $\text{Hd16}$ does not affect the expression pattern of clock-associated genes, it might control flowering time in the photoperiodic pathway without affecting circadian rhythms (Hori et al., 2013). CKI is a member of the highly conserved serine/threonine-specific casein kinases that are related to various signal-transduction processes in eukaryotes (Cheong and Virshup, 2011). The rice CKI gene positively regulates brassinosteroid signaling (Liu et al., 2003) and exerts negative regulation on gibberellin signaling (Dai and Xue, 2010). Moreover, it is reported that the $\text{Hd16/ELI}$ (Kwon et al., 2014) protein can phosphorylate a floral repressor, $\text{Ghd7}$, in vitro (Hori et al., 2013). Another casein kinase gene, $\text{Hd6}$, encoding the CKII α subunit (Takahashi et al., 2001), was also detected as a flowering-time QTL between the temperate $\text{japonica}$ cultivar Nipponbare and an indica cultivar ‘Kasalath’ (reviewed by Hori et al., 2016). CKII is implicated in multiple processes related to development and stress-responses including the circadian clock in plants; however, it belongs to a serine/threonine kinase family that is evolutionarily distinct from CKI (Mulekar and Huq, 2014). Although $\text{Hd6}$ is also not involved in the circadian rhythm, it delays flowering under LD conditions only in the presence of functional $\text{Hd1}$ (Ogiso et al., 2010). Interestingly, the $\text{Hd6}$ gene product does not interact with or phosphorylate the $\text{Hd1}$ gene product in vitro (Ogiso et al., 2010). It has been reported that $\text{Hd16}$ and $\text{Hd6}$ gene products interacted with the $\text{OsPRR37}$ protein in vivo, and phosphorylated different regions of this protein in vitro (Kwon et al., 2015). $\text{Hd2}$ has been detected previously in an $F_2$ population derived from a cross between Nipponbare and Kasalath (Yano et al., 1997; Yamamoto et al., 1998) and isolated as the $\text{OsPRR37}$ gene, an Arabidopsis TOC1 homolog (or a pseudo-response regulator gene) (Koo et al., 2013).

Recently, a genetic resource termed ‘chromosomal segment substitution lines’ (CSSLs) has been developed in rice for detection of QTLs with small effects, and is a set of genetic lines that have distinct genomic fragments introgressed from a recurrent cultivar into a background parent cultivar so as to span the entire region of the genome with the introgressed fragments (Ebitani et al., 2005, Keurentjes et al., 2007). An indica cultivar, ‘Nona Bokra’, showed extremely late flowering compared to the $\text{japonica}$ cultivar Koshiihikari under LD conditions (Uga et al., 2007). CSSLs with Nona Bokra as the donor and Koshiihikari as the recipient background cultivar were developed and QTL analysis was performed for flowering time in the field in Tsukuba, Japan (Takai et al., 2007). Although several flowering-time QTLs have been detected and candidate genes have been proposed for a few of them, it has so far been experimentally confirmed that Nona Bokra has a defective allele of one of the flowering genes, $\text{RFT1}$, due to an amino acid substitution (Ogiso-Tanaka et al., 2013).

In this study, using further analysis of the Nona Bokra–Koshiihikari CSSLs and a set of newly developed nearly isogenic lines (NILs) of the $\text{Hd6}$ and $\text{Hd16}$ genes, we demonstrate that the fine-tuning of critical day length is set by these two CK genes. Mutations in the CK genes have no effect on the rice circadian clocks. Furthermore, we show that $\text{Hd6}$ and $\text{Hd16}$ are involved in the actions of both $\text{Hd1}$ and $\text{Ghd7}$ to control $\text{Ehd1}$, $\text{Hd3a}$, and $\text{RFT1}$. Our results suggest that this fine-tuning of the setting of the critical day length by natural variation in $\text{Hd6}$ and $\text{Hd16}$ may contribute to a potential northward expansion of rice cultivation areas.

Materials and methods

Plant material and growth conditions

We used CSSLs derived from a cross between the $\text{Oryza sativa temperate japonica}$ cultivar Koshiihikari as the recipient and the indica cultivar Nona Bokra as the donor produced by Takai et al. (2007). A set of four new NILs for $\text{Hd16}$ and $\text{Hd6}$ in the genetic background of Koshiihikari were developed, including NILs for $\text{Hd16}$ with the functional allele of $\text{Hd6}$, by crossing ‘Kanto IL5’ by marker-assisted selection (see Supplementary Fig. S1 at JXB online; Hori et al., 2013). Kanto IL5 is one of the isogenic lines derived from crosses between Koshiihikari and Kasalath. It has a 170-kb segment of the Kasalath chromosome around the $\text{Hd6}$ allele in the Koshiihikari background, which means that Kanto IL5 has a functional $\text{Hd6}$ allele of Kasalath (Takeuchi, 2011). We were able to guarantee that the genomic fragment has a single gene mutation to affect flowering-time since this Kasalath fragment had been fine-mapped when these genes were cloned (Takahashi et al., 2001; Hori et al., 2013). In the present work, we used Koshiihikari as the background cultivar. $\text{Hd6}$ was originally cloned as a flowering-time QTL between Nipponbare and Koshiihikari (Hori et al., 2013). Furthermore, there...
is no QTL at the *Hd6* locus between Nipponbare and Koshihikari, indicating that Koshihikari has the same defective allele of *Hd6* as that of Nipponbare. Taken together, it has been proved that the four NILs used in this work have only the *Hd6* and *Hd16* genes to cause differences in flowering-time. We also used a set of NILs for *Hd16* and *Hd1*, described in Hori et al. (2013). Plants were grown in a growth chamber under various photoperiod conditions (10, 12.5, 13, 13.5, or 14.5 h); light was provided by metal halide lamps (ca. 450 μmol m⁻² s⁻¹). Temperature was maintained at 28/24 °C according to the day–night cycle. Light pulses in night-break experiments were provided using a red LED panel (intensity of 12.5 μmol m⁻² s⁻¹) for 15 min at the mid-point of the dark period. The flowering time was recorded as the time (or date) when the first panicle emerged.

Expression and sequencing analyses

Total RNA was extracted from leaves using TRIZOL (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized after treatment with DNase I (Nippongene). The gene-specific primers and TaqMan probe sequences were the Power SYBR Green protocol (ABI) on an ABI PRISM 7900 was carried out using the TaqMan PCR protocol (Nippongene) or sized after treatment with DNase I (Nippongene) from 5 μg of total RNA using Superscript II RTase (Invitrogen). Realtime qRT-PCR was carried out using the TaqMan PCR protocol (Nippongene) or the Power SYBR Green protocol (ABI) on an ABI PRISM 7900 Sequence Detection System according to the manufacturer's instructions. The gene-specific primers and TaqMan probe sequences were as described previously (Nemoto et al., 2016). A rice ubiquitin gene (Oso2g0161900) was used for normalization. *Hd2* gene expression was analysed, and the PCR primers and TaqMan probes were as follows: *Hd2*-F, 5´-CAGAAAAGGAAAGAGCGCAAC-3´; *Hd2*-R, 5´-CTGCTCGGCCAGCCTC-3´; TaqMan probe, 5´-TCGGAAAAGAAGGTGCCTGACAGGAG-3´. We determined the sequences of the PCR products of *Hd16* and *Hd6* expressed in Nona Bokra CDNA by using primers designed from Nipponbare and Kasalath cDNA sequences, respectively (see Supplementary Fig. S2).

Results

The essential genes for flowering under LD conditions in Nona Bokra are located on chromosomes 3 and 6

In this study, we used a subset of CSSLs carrying chromosomal segments from Nona Bokra in the Koshihikari genetic background, which were developed by Takai et al. (2007). First, we selected nine CSSLs that exhibited a severe late-flowering phenotype under natural field conditions in Tsukuba (36° N; Takai et al., 2007). Compared with previous studies using populations of a cross between Nipponbare and Kasalath (reviewed by Yano et al., 2001), the selected CSSLs have introgressed fragments located on chromosome 3 (*Hd9*, *Hd8*, and *Hd6*), chromosome 6 (*Hd3a*, *Hd3b*, and *Hd1*), chromosome 7 (*Hd4* and *Hd2*), and chromosome 10 (*Hd14*) (Fig. 1A, Yano et al., 2001). Then, we grew the CSSLs under 10 h light (L)/14 h dark (D) and 14.5 h L/9.5 h D conditions to examine their flowering times (Fig. 1B). Under 10 h L/14 h D conditions, all lines flowered at ~50 d after sowing, with no significant differences in flowering time among the recurrent parents (Koshihikari and Nona Bokra types) and the CSSLs tested (Fig. 1B).

Under the 14.5 h L/9.5 h D conditions, Nona Bokra showed no flowering during the test period, whereas Koshihikari flowered at ~78 d after sowing. All the tested CSSLs exhibited significantly later flowering than that of Koshihikari (Fig. 1B). We found that two CSSLs, SL508 and SL519, were similar to Nona Bokra, as they showed no flowering under 14.5 h L/9.5 h D conditions up to 200 d after sowing. The majority of chromosomes 3 and 6 were substituted with Nona Bokra chromosomal segments in SL508 and SL519, respectively (Fig. 1A). This result suggested that the essential genes for the non-flowering phenotype under 14.5 h L/9.5 h D conditions are located independently on chromosomes 3 and 6 of Nona Bokra.

The rice florigen gene *RFT1*, located on chromosome 6, is a major floral activator under 14.5 h L/9.5 h D conditions (Komiya et al., 2009). Nona Bokra has a defective allele of *RFT1*; therefore, only *Hd3a* functions as a florigen in this variety (Ogiso-Tanaka et al., 2013). As SL519 has a Nona Bokra-type chromosome 6, it possesses non-functional *RFT1*. Although SL520 also has non-functional *RFT1* (Ogiso-Tanaka et al., 2013), it flowered at ~130 d after sowing under 14.5 h L/9.5 h D conditions (Fig. 1B). This result was consistent with the fact that *RFT1* RNAi plants exhibit delayed flowering under the same photoperiod conditions (Komiya et al., 2009), implying that the other florigen gene, *Hd3a*, might not function appropriately in SL519. Therefore, some unknown but critical natural variation inhibiting the activity of *Hd3a* might exist in the Nona Bokra segment in SL519. Although many of the Nona Bokra-type chromosomal segments described above contain previously identified flowering-time QTLs such as *Hd2* on chromosome 7, no molecular analyses at the level of nucleotide polymorphism have been performed except for the *RFT1* gene (Ogiso-Tanaka et al., 2013).

Key QTLs for recognition of critical day length are located on the long arm of chromosome 3 in Nona Bokra

To examine the effect of Nona Bokra alleles on the setting of the critical day length, we examined *Hd3a* expression in selected CSSLs and the Koshihikari and Nona Bokra parents in the morning (i.e. 2 h after lights on) after a 6-d entrainment period under various day-length conditions (Fig. 2A). We found that the photoperiodic profile of *Hd3a* expression differed markedly in the Koshihikari and Nona Bokra types, with high expression levels of *Hd3a* being detected under 10 h L/14 h D conditions, whereas low levels were observed under 14.5 h L/9.5 h D conditions for all the tested lines. However, the pattern of the decrease in rate of *Hd3a* expression in relation to increasing photoperiod differed significantly in Koshihikari, Nona Bokra, and the tested CSSLs. We plotted these data with both logarithmic and normal axes (Fig. 2A). The peak values of *Hd3a* expression under 10 h L/14 h D conditions displayed similar ranges among the tested lines, and gene expression showed dynamic ranges of more than three orders of magnitude. In the case of Koshihikari, the expression level decreased gradually across photoperiods of 10–13.5 h but rapidly between photoperiods of 13.5 and 14.5 h. On the other hand, expression in Nona Bokra decreased proportionately photoperiods of 10–14.5 h. The expression level of *Hd3a* under 14.5 h L/9.5 h D conditions was ~10⁻⁵–10⁻⁶, which is around the detection limits of this quantitative RT-PCR analysis (Fig. 2A). The decreasing profiles of the tested CSSL lines could be classified into
three patterns: the lines SL509, SL519, SL520, and SL523 belonged to the Koshihikari type; the lines SL508, SL510, and SL537 belonged to the Nona Bokra type; and the lines SL521 and SL526 exhibited intermediate patterns between the Koshihikari and Nona Bokra types. The SL508 and SL510 lines, which contained genomic fragments of chromosome 3 from Nona Bokra, exhibited effects that were as strong as those of the Nona Bokra type, and the two previously identified flowering-time QTLs, \(Hd6\) and \(Hd16\), were located on the introgressed fragments of these two lines; therefore, we focused on SL508 and SL510 in this study. There is no candidate gene isolated yet for SL537.

We compared the related flowering-time gene expression patterns in SL508, SL510, and their donors (Fig. 2B). SL508 and SL510 exhibited gene expression of \(Ehd1\), \(Hd3a\), \(Ghd7\), and \(RFT1\) that was similar to that of Nona Bokra (Fig. 2B). SL508 contains the majority of chromosome 3 from Nona Bokra (Fig. 1A), whereas SL510 contains only the lower part of chromosome 3 from this variety. QTL analyses of heading time using an F2 population derived from a cross between the Koshihikari and Nona Bokra types (Uga et al., 2007) demonstrated that the candidate QTL contained the \(Hd6\) region on the lower part of chromosome 3. \(Hd6\), originally identified as a QTL using the Nipponbare and Kasalath cultivars,
Fig. 2. Expression analysis of CSSLs and their parents under various photoperiods. After a 6-d entrainment period, the fully expanded leaves were harvested from three replicates composed of two or three independent plants at 2 h after lights-on. (A) Expression of Hd3a under different photoperiods of nine CSSLs and their parents. The level of gene expression is shown on a normal (upper) and a log scale (lower). (B) The CSSLs were selecting according to the contribution of chromosome 3 from Nona Bokra and the expression patterns of Ehd1, Hd3a, and RFT1 that were similar to that of Nona Bokra. Each value is the average of three biological replicates (means ±SD).
encodes the alpha subunit of CK II. Nipponbare contains the non-functional allele, *hd6*, due to a non-synonymous change, whereas Kasalath contains a functional *Hd6* allele (Takahashi et al., 2001). Koshihikari has the same non-functional *Hd6* allele as Nipponbare, whereas Nona Bokra might have a functional *Hd6* allele, similar to the Kasalath type (Uga et al., 2007). The *Hd16* QTL for heading time, which is in the vicinity of *Hd6* on chromosome 3, was detected in a cross between the Nipponbare and Koshihikari types (Matsubara et al., 2008). *Hd16* was shown by map-based cloning to encode a casein kinase-I protein (Hori et al., 2013). Thus, we focused on *Hd16* and *Hd6* as candidate fine-tuning genes for setting the critical day length for *Hd3a* expression in rice. Since it is already known that *Hd6* and *Hd16* in Koshihikari have defective mutations (Takahashi et al., 2001; Hori et al., 2013), in this current work we sequenced these genes in Nona Bokra and found that both of them appear to be the functional alleles (see Supplementary Fig. S2).

Casein kinase I and II are involved in setting the genes for critical day length

To elucidate the contribution of the *Hd16* and *Hd6* genes in setting the critical day length, we produced a series of four nearly isogenic lines (NILs) containing all possible combinations of the defective and functional alleles of *Hd16* and *Hd6* (originated from Nipponbare and Kasalath alleles, respectively) in the genetic background Koshihikari (Hori et al., 2013; Fig. 3A). Notably, *Hd16* and *Hd6* are closely linked genetically, and the physical distance between the two genes is ~1.5 Mb. We investigated the effects of the *Hd16* and *Hd6* alleles on the flowering phenotype under 10-, 12.5-, 13.5-, and 14.5-h photoperiod conditions (Fig. 3B). Under 10-h photoperiod conditions, plants exhibited slightly earlier flowering when both the *Hd16* and *Hd6* alleles were functional; conversely, they displayed markedly later flowering under a 14.5-h photoperiod (Fig. 3B). Under 12.5 h L/11.5 h D conditions, all lines flowered at almost the same time, which was inconsistent with the gene expression patterns of related genes at their seedling stages (Fig. 3C, D). This result implied that the gene expression patterns at this developmental stage might not be correlated directly with their flowering time. Under 13.5 h L/9.5 h D conditions, late flowering was observed in plants with the functional allele of *Hd16*. Comparing the functions for the two alleles, *Hd16* had the stronger effect for flowering under this photoperiod. Under 14.5 h L/9.5 h D conditions, the functional alleles of both *Hd16* and *Hd6* contributed significantly to a delay in flowering, and each gene functioned additively to control flowering time (Fig. 3B). These results indicated that the functional alleles of *Hd16* and *Hd6* were related to both promotion and repression of flowering time under the 10- and 14.5-h photoperiods, respectively.

Next, we examined the gene expression pattern of *Hd3a* in the morning with varying day lengths (Fig. 3C). The results indicated that both *Hd6* and *Hd16* are major determinants of critical day length for *Hd3a* gene expression. We further examined the gene expression of related flowering-time genes (Fig. 3D). The expression patterns of *Ghd7* were similar among these lines, with expression increasing with longer photoperiods. Only the *Hd16Hd6* line displayed a Nona Bokra-type in the expression of *Ehd1*, *Hd3a*, and *RFT1*. *hd16Hd6* and *Hd16Hd6* showed intermediate patterns between Koshihikari and Nona Bokra. The *hd16hd6* line exhibited gene expression patterns of *Ehd1*, *Hd3a*, and *RFT1* that were similar to those of the Koshihikari type (Fig. 3D). These results indicated that the functional alleles of *Hd16* and *Hd6* that were necessary for the rapid decrease depended on longer photoperiods, in a manner similar to the Nona Bokra type. Significant repressions of *Hd3a* and RFT1 gene expression were observed between the 13- and 13.5-h photoperiods, but not at the 10-, 12-, and 14.5-h ones, for the *hd16Hd6* and *Hd16hd6* lines; in contrast, significant repressions were observed at the 12.5-, 13-, and 13.5-h photoperiods, but not at 10- and 14.5-h ones for the *hd16hd6* line. These results indicated that the combination of *Hd16* and *Hd6* alleles fine-tuned the critical day length for florigen gene expression, which might determine appropriate cultivation areas by setting flowering times (or heading dates) under certain environments.

The effects of *Hd16* and *Hd6* on diurnal expression patterns of flowering-related genes

We examined the diurnal expression patterns of flowering-time genes at intervals of 2 h in the dark or 3 h in the light for one day using 3-week-old seedlings of NILs grown under conditions of 12.5 h L/11.5 h D (Fig. 4 left panel) and 13.5 h L/10.5 h D (Fig. 4 right panel). The diurnal expression patterns of *Hd1*, *Hd2*, and *Ghd7* were not affected by the *Hd16* or *Hd6* alleles. *Ehd1* expression was activated only with the *Hd16* functional allele during the early night period from 0.5 to 4.5 h after lights-off under 12.5 h L/11.5 h D conditions. Thus, *Hd16* could function as an *Ehd1* activator in the early night regardless of *Hd6* function. In addition, under 12.5 h L/11.5 h D conditions, the florigen genes *Hd3a* and *RFT1* expressed a similar diurnal pattern; however, the expression level of *Hd3a* was higher than that of *RFT1*. At 2 h after lights-on, *Hd3a* expression was slightly repressed by the functional alleles of both *Hd16* and *Hd6*. *RFT1* expression was also slightly repressed by *Hd16* and *Hd6* from 2 to 5 h after lights-on. Taking these findings together, under 12.5 h L/11.5 h D conditions, *Hd16* seems to have a dual function for flowering genes as a promoter at night (independent of *Hd6*) and as a repressor in the morning (dependent on *Hd6*). Under 10 h L/14 h D conditions, the expression levels of *Ehd1*, *Hd3a*, and *RFT1* were similar in the morning (Fig. 3D), but slightly lower only in the *Hd16Hd6* lines. We further examined the expression level of *Ehd1* at 1 h before and 5 h after lights-off (see Supplementary Fig. S3) under 10 h L/14 h D conditions, and observed activation of *Ehd1* by *Hd16*. Thus, *Hd16* might function as a floral promoter under 10 h L/14 h D conditions. We next examined gene expression with NILs grown under 13.5 h L/10.5 h D conditions (Fig. 4 right panel). These genes were synergistically repressed by both *Hd16* and *Hd6*. The diurnal expression patterns of *Hd1*, *Hd2*, and *Ghd7* were not affected by *Hd16* or *Hd6*, and showed patterns similar to those observed under 12.5 h L/11.5 h D conditions. On the
Fig. 3. Comparison of flowering time and photoperiodic response in NILs of Koshihikari. (A) Graphical representation of the NIL genotypes. Functional Hd16 and Hd6 were introduced from Nipponbare and Kanto IL5, respectively. The Hd6 allele of Kanto IL5 was originated from Kasalath (Takeuchi, 2011). (B) Days to flowering of plants grown under various photoperiods after sowing. Data are means ±SD for 6−10 plants per genotype. NIL genotypes are indicated. (C, D) Three-week-old NIL plants were entrained for 6 d with various photoperiods and expression patterns of Hd3a, Ehd1, Ghd7, and RFT1 are shown. Data for Hd3a are shown on a normal scale (C) and on log scale (D). The experimental procedures were the same as those described in Fig. 2.
other hand, the gene expression levels of *Ehd1*, *Hd3a*, and *RFT1* were highly repressed by either *Hd16* or *Hd6* and by the combination of *Hd16* and *Hd6* (Fig. 4 right panel). In these experiments, *Hd16* had stronger repression activity than *Hd6* (Fig. 4 right panel). These results were roughly consistent with the flowering phenotypes, in that the functional *Hd16* caused a slight flowering delay under 13.5 h L/10.5 h D conditions (Fig. 3B).
Hd16 did not enhance Ghd7 function induced by night-break under a 10-h photoperiod

The Hd16 recombinant protein (rHd16) phosphorylates rGhd7 specifically in vitro (Hori et al., 2013), and phosphorylation of Ghd7 has been thought to enhance its repressive activity. To verify whether Ghd7 repression activity was enhanced by Hd16 through phosphorylation, we performed a series of night-break experiments to induce Ghd7 transcription by introducing light pulses at night (Itoh et al., 2010) in the related CSSL lines (Fig. 5A) and the Hd16 Hd6 NILs (Fig. 5B). We then examined the repression level of Hd3a in the morning following the night-break treatment. We observed that the night-break effect for Hd3a was affected by the function of Hd16, but was not dependent on the Hd6 genotype (Fig. 5B). After a night-break, the expression of Hd3a was lower in plants with the non-functional hd16 allele than in those with the functional Hd16 allele, as indicated by the fact that the night-break repression among the tested lines was clearest in the hd16hd6 line. This finding was contrary to our prediction that the night-break-induced Ghd7 gene product might be phosphorylated by Hd16, i.e. that its repression activity might be enhanced with the functional Hd16 allele. An investigation of the night-break effect on the CSSLs and their donors (Fig. 5A) showed results that were consistent with those found in the NILs. The Hd3a expression level after a night-break was more repressed with the non-functional hd16 allele than with the functional Hd16 allele. Therefore, we propose that Hd16 may function as a floral promoter under 10 h L/14 h D conditions, independent from the Ghd7 repressor function. The Ghd7 gene product induced by a night-break under 10 h L/14 h D conditions might not be phosphorylated in vivo by Hd16. Alternatively, the phosphorylated Ghd7 gene product might not function as a repressor. Taken together, our results indicate that Hd16 is unrelated to the night-break repression mediated by Ghd7, and it can induce Hd3a and RFT1 independently at night under 10 h L/14 h D conditions.

Hd16 seems to enhance Hd1 activator and repressor functions under SD and LD conditions, respectively

Previously, we reported that Ehd1 was activated by Hd1 early during the night period under 10 h L/14 h D conditions (Nemoto et al., 2016). In the present study, we found that Ehd1 was activated by Hd16 early during the night under 12.5 h (Fig. 4 left panel) and 10 h (see Supplementary Fig. S3) photoperiod conditions. These findings led us to investigate whether Hd16 was required for Ehd1 activation by Hd1. Using a series of NILs for the Hd1 and Hd16 alleles in the Koshihikari genetic background produced by Hori et al. (2013), we assessed the expression levels of Ehd1 before and after lights-off under 10 h L/14 h D conditions. High levels of Ehd1 transcript were observed at 5 h after lights-off in the Hd1Hd16 line, while low levels were found in the other lines (Fig. 6A). This result indicated that Ehd1 activation by Hd1 requires Hd16 function. Hd1 has a bifunctional role for flowering, which is switched by differing photoperiods: it functions as a floral promoter under SD conditions and as a floral repressor under LD conditions. Thus, we further examined whether the repression activity of Hd1 required Hd16 under 14.5 h L/9.5 h D conditions. NILs on Hd1Hd16 were grown under 13.5 h L/10.5 h D conditions for 3 weeks and then examined for gene expression of Ehd1 and Hd3a at 2 h after lights-off (Fig. 6B). The expression levels of Ehd1 and Hd3a were repressed by Hd1. Under 13.5 h L/9.5 h D conditions, Hd16 seemed to enhance Hd3a repression activity by Hd1. In addition, Hd16 seemed to repress Ehd1 in the presence of the defective Hd1 allele. These results suggest that Hd16 functions to enhance the Hd1 repressor activity even under LD conditions. rHd1 protein is not phosphorylated by rHd16 protein (Hori et al., 2013); thus, indirect mechanisms may be involved in the enhancement of Hd1 repressor activity.

Discussion

QTLs essential for LD flowering are located on chromosomes 3 and 6 in Nona Bokra

The rice indica cultivar Nona Bokra, which originated in India, exhibits a strong photoperiodic response. QTL analyses for flowering time were carried out with an F2 population derived from a cross between Nona Bokra and Koshihikari (Uga et al., 2007), and the CSSLs were derived from a back-cross between Nona Bokra as the donor and Koshihikari as the recipient cultivar (Takai et al., 2007). All QTLs with Nona Bokra alleles contributed to late flowering under
natural field conditions in Tsukuba, Japan. Here, we study a selected set of the CSSLs under artificially controlled photo-period conditions.

Two lines, SL508 and SL519, showed no flowering even at 180 d under 14.5 h L/9.5 h D conditions (Fig. 1B). A major QTL for late flowering was identified on SL519 as a natural variation in the \( RFT1 \) gene (Ogiso-Tanaka et al., 2013). Further analysis was needed on SL519, as SL508 contained the entire region of chromosome 3 from Nona Bokra (Fig. 1A). The flowering-time phenotypes of the related CSSLs SL509 and SL510, which each contained approximately half of the segments of chromosome 3, suggested that critical genetic interactions occur between distinct QTLs to confer non-flowering phenotypes under LD conditions.

This study focused on \( Hdl6 \) and \( Hd6 \) located on chromosome 3, in which the background parent cultivar, Koshihikari, has both the defective alleles of \( Hd6 \) and \( Hdl6 \), and we analysed them using a series of newly developed NILs. We demonstrated that both \( Hdl6 \) and \( Hd6 \) genes contributed to the fine-tuning of the setting of the critical day length (Fig. 3B–D). This is the first study to identify genetic determinants for changing the critical day length setting in photoperiodic flowering.

Hd16 and Hd6 contribute to fine-tuning of the setting of the critical day length in rice photoperiodic flowering

The sensitive and accurate threshold control of \( Hd3a \) expression to set the critical day length in rice is controlled by two distinct gating mechanisms through \( Ehdl \) and \( Ghd7 \) (Itoh et al., 2010). The fine-tuning of the setting of the critical day length in \( Lemna \) flowering may determine its natural growth habitats (Yukawa and Takimoto, 1976; Beppu and Takimoto, 1981). Thus, it is possible that fine-tuning systems are required for setting the critical day length for florigen expression in natural variants of rice. The critical day length is apparently determined by both the expression levels of florigen genes in the leaves and the sensitivity for florigen proteins at the apex region. It has been reported that young rice seedlings can clearly induce expression of the \( Ehdl \), \( Hd3a \), and \( RFT1 \) genes according to the day lengths to which plants are subjected, but the expression of these florigen genes at the developmental stages is not able to determine flowering-time, perhaps due to immature perception systems for the florigen proteins at the apex, and/or low peak levels of florigen gene expression. The threshold of florigen gene expression required to induce floral transition depends on the developmental stage at which the plants are tested, and then for a given stage we can experimentally determine the critical day length. Expression levels of florigen genes even at the young developmental stage were critically regulated by day length, suggesting that plants at this stage can be targeted to elucidate critical day-length recognition in terms of gene regulation. In this study, we found that \( Hdl6 \) and \( Hd6 \) contribute to the fine-tuning of the setting of this critical day length for florigen gene expression in rice. \( Hdl6 \) and \( Hd6 \) encode CKI and CKII, respectively, which are members of an evolutionally conserved serine/threonine kinase family. \( Hdl6 \) and \( Hd6 \) are not involved in the circadian clock system in rice (Ogiso et al., 2010; Hori et al., 2013). It is still possible that phosphorylation of target transcriptional protein complexes not related to circadian clock systems may enhance the transcriptional activation and/or repression of florigen genes.

The natural variation of \( Hd6 \) and \( Hdl6 \) were investigated in cultivars of \textit{temperate japonica}, a rice subspecies adapted to northern temperate regions with longer photoperiods in...
Hd16 and Hd6 might function as a floral promoter under 10-h photoperiod conditions

The flowering-time phenotypes of rice grown under field conditions at Tsukuba, Japan, could be mimicked under the longer photoperiod conditions applied in artificially controlled growth chambers. As Hd16 and Hd6 were first isolated as floral repressors under natural field conditions after QTL cloning, this study investigated their effects on flowering phenotypes and related gene expression under artificially controlled 10-h photoperiod conditions in growth chambers, which simulate typical SD conditions.

Slightly early-flowering phenotypes were exhibited when both Hd16 and Hd6 alleles were functional (Fig. 3B). Thus, Hd16 and Hd6 seemed to act as a floral promoter under 10-h photoperiod conditions. Consistently, we found that both Hd16 and Hd6 together could activate Ehd1 and Hd3a (weak activation) at night (see Supplementary Fig. S3) under 10-h photoperiod conditions. Under longer photoperiods, such as 12.5 h, the promotion of flowering disappeared (Fig. 3B). In addition, Hd16 could activate Ehd1, but not Hd3a or RFT1, during the early night period under 12.5-h photoperiod conditions regardless of the Hd6 genotype (Fig. 4 left panel). These results imply that Hd16 may work as an activator of rice flowering in the presence of relatively short photoperiods. Taking these findings together, Hd16 and Hd6 might function as a floral promoter under SD conditions, although RFT1 expression in the morning was not clearly affected by Hd16 and Hd6 (Fig. 3D). The promotion of activity of related genes by Hd16 was more robust than that of Hd6 under SD conditions; however, earlier flowering and greater Hd3a expression were observed only with both Hd16 and Hd6 functional alleles.

Hd16 assists Hd1 activity under both LD and SD conditions

Previously, we reported that Hd1 activates Ehd1 regardless of Ghd7 genotype during the night under 10-h photoperiod conditions (Nemoto et al., 2016). In addition, both Hd16 and Hd6 together may have a bifunctional action to control flowering time, similar to Hd1. These results led us to examine whether Ehd1 activation by either Hd1 or Hd16 occurred independently of each other. The results showed that Hd16 and Hd1 work together to activate Ehd1 under 10-h photoperiod conditions (Fig. 7B). Furthermore, Hd1 activated Hd3a expression after lights-off under 10-h photoperiod conditions (see Supplementary Fig. S5). Activation of Hd3a expression was also enhanced by functional Hd16 (Supplementary Fig. S5). These results suggest that Hd16 can enhance Hd3a expression at night under 10-h photoperiod conditions through the function of Hd1 (Supplementary Figs S4 and S5).
Under 13.5-h photoperiod conditions, Hdl repressed Ehd1 and Hdl6a in the morning (Fig. 6B). Interestingly, Hdl6 also repressed Hdl3a expression, which required the functional Hdl allele, but not Ehd1 (Fig. 6B). On the other hand, Hdl6 repressed Ehd1 expression with the defective hdl allele (Fig. 6B). These results implied that Ehd1 and Hdl3a repression by Hdl6 might occur through Ghd7 phosphorylation. Notably, rHd1 can phosphorylate rGhd7 in vitro (Hori et al., 2013), and Ghd7 can interact with Hdl to repress Ehd1 and Hdl3a (Nemoto et al., 2016). Taking these findings together, Hdl6 seems to support the functional enhancement of Hdl activity, which can change between activator and repressor under certain photoperiod conditions. In addition, rHd1 is not phosphorylated by rHd16 and rHd6 in vitro (Ogiso et al., 2010; Hori et al., 2013). However, Hdl6 and Hdl6 interact with the Hdl2/OsPRR37 protein in vivo, and different regions of Hdl2/OsPRR37 are phosphorylated (Kwon et al., 2015); rHd16 phosphorylates rGhd7 in vitro (Hori et al., 2013). The diurnal expression patterns of Hdl2/OsPRR37 and Ghd7 were not affected by Hdl6 and Hdl6 under 12.5- and 13.5-h photoperiod conditions (Fig. 4). These results imply that the activity of Hdl2/OsPRR37 and Ghd7 gene products might be regulated by post-translational modification by Hdl6 and Hdl6 together to control florigen genes in rice, partially through the physical interaction between Hdl1 and Ghd7 gene products (Nemoto et al., 2016). Hdl2 functioned to delay flowering under the 14.5-h photoperiod and caused significantly later flowering when the functional allele of Hdl1 was present (Hori et al., 2013). Future studies are required to determine whether Hdl1 can interact physically with Hdl2 in vivo to control downstream genes such as Ehd1, Hdl3a, and RFT1.

Supplementary data
Supplementary data are available at JXB online.

Fig. S1. Graphical representations of the genotypes of the NILs Hdl6/Hdl6 and hdl6/Hdl6 with SSR markers.

Fig. S2. Alignments of Hdl6 and Hdl6 coding sequences and deduced amino acid sequences.

Fig. S3. The non-functional allele of hdl6 showed higher repression of Ehd1 expression than the functional Hdl6, independent of Hdl6 function, under a 10-h photoperiod.

Fig. S4. Illustration of Hdl3a expression regulated by Hdl6 and Hdl6 in the morning and at night under various photoperiod conditions.

Fig. S5. Expression of Hdl3a in NILs at night under a 10-h photoperiod.

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Author contributions
TI conceived and designed all the experiments in this work; YN performed most of the experiments; KH prepared a series of NIL lines; YN and TI wrote the manuscript and KH revised it.

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