Natural Variation in Hd17, a Homolog of Arabidopsis ELF3 That is Involved in Rice Photoperiodic Flowering

Kazuki Matsubara1,2, Eri Ogiso-Tanaka1, Kiyosumi Hori1, Kaworu Ebana1, Tsuyu Ando1 and Masahiro Yano1,*

1National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, 305-8634 Japan
2Present address: National Institute of Crop Science, Tsukuba, Ibaraki, 305-8518 Japan

*Corresponding author: E-mail, myano@affrc.go.jp; Fax, +81-29-838-6070

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Flowering time of rice depends strongly on photoperiodic responses. We previously identified a quantitative trait locus, Heading date 17 (Hd17), that is associated with a difference in flowering time between Japanese rice (Oryza sativa L.) cultivars. Here, we show that the difference may result from a single nucleotide polymorphism within a putative gene that encodes a homolog of the Arabidopsis EARLY FLOWERING 3 protein, which plays important roles in maintaining circadian rhythms. Our results demonstrate that natural variation in Hd17 may change the transcription level of a flowering repressor, Grain number, plant height and heading date 7 (Ghd7), suggesting that Hd17 is part of rice's photoperiodic flowering pathway.

Keywords: EARLY FLOWERING 3 (ELF3) • Heading date 17 (Hd17) • Map-based cloning • Natural variation • Photoperiodic flowering • Rice.

Introduction

Photoperiodic control of flowering is one of the most significant components of the interaction between plants and their environment (Thomas and Vince-Prue 1997). Recent advances in this field have demonstrated that external light signals perceived by photoreceptors activate a florigen-type promoter, which is mediated in part by the circadian clock, thereby promoting flowering in response to favorable daylength in both monocots and eudicots (Song et al. 2010, Tsuji et al. 2010).

By analyzing natural variation in flowering time, several quantitative trait loci (QTLs) with large effects have been successfully isolated, and this knowledge has improved our understanding of the genetic control of rice flowering (Yano et al. 2000, Takahashi et al. 2001, Kojima et al. 2002, Doi et al. 2004, Xue et al. 2008, Itoh et al. 2010, Ogiso et al. 2010, Wei et al. 2010, Yan et al. 2011). Recently, we identified a new QTL, Heading date 17 (Hd17), that is associated with a small difference in flowering time [<3 d under natural day (ND) conditions] between two Japanese rice (Oryza sativa L.) cultivars, ‘Nipponbare’ and ‘Koshihikari’ (Matsubara et al. 2008). In the present study, we demonstrated that Hd17 encodes a homolog of Arabidopsis EARLY FLOWERING 3 (ELF3), which plays important roles in maintaining circadian rhythms (Dixon et al. 2011, Nefissi et al. 2011, and references therein), and that the QTL lies within the pathway responsible for rice's photoperiodic flowering through the modulation of the transcription level of a flowering repressor, Grain number, plant height and heading date 7 (Ghd7), but probably not through affecting circadian rhythms.

Results and Discussion

We found no significant difference in days to flowering between a near isogenic line (NIL) carrying Hd17 from ‘Koshihikari’ (NIL-Hd17, 45.3 ± 1.5 d) and the recurrent parent ‘Nipponbare’ (45.6 ± 1.5 d) under short-day (SD) conditions (10 h light/14 h dark), but NIL-Hd17 (97.8 ± 2.8 d) was found to flower about 8 d later than ‘Nipponbare’ (89.6 ± 2.6 d) under long-day (LD) conditions (14.5 h light/9.5 h dark) (Fig. 1A). In addition, we compared days to flowering, culm length, panicle length, panicle number per plant, plant weight, panicle weight per plant, and stem and leaf weight between ‘Nipponbare’ and NIL-Hd17 under ND conditions (Fig. 1B, Supplementary Fig. S1). Among these traits, only days to flowering and panicle length were significantly greater in NIL-Hd17 than in ‘Nipponbare’, but the difference was small. Thus, ‘Nipponbare’ and NIL-Hd17 do not differ greatly in any trait other than flowering time under ND and LD conditions.

Previously, we mapped Hd17 within the 328 kb genomic region between the molecular markers P548D347 and...
0007O20 on chromosome 6, near two rice FT-like genes, RFT1 and Hd3a (Matsubara et al. 2008; Fig. 1C). Based on these results, we attempted positional cloning of Hd17. First, we carried out fine mapping using a BC2 segregating population consisting of 500 plants. Hd17 was located between the markers InDel_1 and CAPs_1 (Fig. 1C). Then, we generated a high-resolution map for 3,120 BC2 plants. A high-resolution linkage analysis demonstrated that Hd17 was delimited within a 43.2 kb genomic region between two markers, SNP_3 and SNP_6. Three markers (SNP_4, SNP_5 and SNP_6) co-segregated with the Hd17 phenotype (Fig. 1C, Supplementary Fig. S2, Supplementary Table S1). In this candidate region, 10 putative open reading frames (ORFs) were predicted in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp). Comparison of the sequence of the candidate region between the ‘Nipponbare’ and ‘Koshihikari’ genomes revealed that Os06g0142600 (a homolog of the Arabidopsis ELF3 protein; Supplementary Fig. S3) and Os06g0142650 (protein kinase; Supplementary Fig. S4A) possess a single nucleotide polymorphism (SNP) within their ORFs (Fig. 1C).

It has been proposed that Arabidopsis ELF3 acts as a transcriptional regulator of clock-associated genes such as LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and GIANTANEA (GI); a clock-output gene, CHLOROPHYLL A/B BINDING 2 (CAB2); and a floral inducer such as CONSTANS (CO) (Covington et al. 2001, Hicks et al. 2001, Liu et al. 2001, Kim et al. 2005, Yu et al. 2008, Yoshida et al. 2009, Dixon et al. 2011, Nefissi et al. 2011). In addition, the SNP in Os06g0142600 resided within a highly conserved region between the rice and Arabidopsis genomes. These implications motivated us to test whether the SNP caused the flowering time difference. Therefore, we transformed the corresponding ‘Koshihikari’ genomic fragment into ‘Nipponbare’ by means of Agrobacterium-mediated transformation. The fragment consisted of a 2.9 kb upstream sequence, the putative coding region (approximately 4.4 kb) and a 2.1 kb downstream sequence bounded by XhoI and BamHI (Fig. 1C).

We then grew T1 progeny derived from single-copy T0 transformants under LD conditions, and scored their flowering time.

Fig. 1 Continued

are means ± SD (n = 20). Differences between means were compared using the two-tailed Student’s t-test. (B) ‘Nipponbare’ and NIL-Hd17 grown under natural day (ND) conditions after flowering. (C) Linkage analysis and map-based cloning of Hd17. Hd17 consists of four exons and three introns. (D) Days to flowering of two independent T1 segregants of the Hd17 transgene from ‘Koshihikari’ under LD conditions. Differences in days to flowering between plants with and without the transgene were compared using the Wilcoxon rank-sum test. (E) Comparison of transcription levels of Hd17 mRNA between ‘Nipponbare’ and NIL-Hd17 under LD conditions. Values are means ± SD (n = 3). ZT, Zeitgeber time. Leaf samples were collected at 60 d after germination.
The result revealed that T1 plants with the transgene tended to flower later than those without the transgene, and the difference was significant in one of the two progeny (but marginally significant in the other one) (Fig. 1D). It should be noted that the phenotypic difference was incompletely expressed, which is the nature of a QTL with a small effect. On the other hand, it seems that the annotated protein kinase (Os06g0142650) is not a causal factor of the flowering time difference, since the non-synonymous amino acid change is not present at a position within a putatively critical domain in the protein (Supplementary Fig. S4A). In addition, RNA interference with expression of the gene did not significantly affect flowering time (Supplementary Fig. S4B). These results strongly suggested that Os06g0142600 is the Hd17 gene, and that the SNP in Os06g0142600 caused the flowering time difference between 'Nipponbare' and NIL-Hd17. Nevertheless, the nucleotide change in Hd17 does not appear to affect its transcription level [a < 1.9-fold change at the maximum, which occurred at Zeitgeber time (ZT) 20; Fig. 1E].

Arabidopsis ELF3 regulates circadian rhythms by affecting the transcription of clock-associated genes such as LHY, CCA1 and GI, and is followed by transcription of downstream genes such as CO. It has also been reported that rice conserves a clock function (Sugiyama et al. 2001, Murakami et al. 2007). Therefore, we examined the effect of natural variation in Hd17 on transcription levels of the rice homologs of the Arabidopsis clock-associated genes, OsLHY (CCA1), OsPRR1 (TOC1/PRR1) and OsGI (GI) (Ogiso et al. 2010), and of two floral repressors, Hd1 (CO) and Ghd7 (no homolog in the Arabidopsis genome), under LD conditions (Yano et al. 2000, Xue et al. 2008). Under these conditions, the flowering time difference between 'Nipponbare' and NIL-Hd17 was evident.

For the clock-associated genes, no clear difference was observed in their transcription levels between 'Nipponbare' and NIL-Hd17 (Fig. 2), and these results were supported by our observation that the pattern of rice CAB1R (CA2) gene expression (for periods of free-running rhythm) was unlikely to be affected by the nucleotide change (Supplementary Fig. S5). On the other hand, although there was no obvious difference in the level of Hd1 (CO) transcripts between 'Nipponbare' and NIL-Hd17, the level of Ghd7 in 'Nipponbare' was generally lower than that in NIL-Hd17 (a > 2.5-fold change at the peak, which occurred at ZT 3; Fig. 2). This observation is supported by the fact that transcription levels of three downstream floral inducers—Early heading date 1 (Ehd1; with no homolog in the Arabidopsis genome; Doi et al. 2004), Hd3a and Rice FT-like 1 (RFT1)—and of Ghd7 were higher in 'Nipponbare' than in NIL-Hd17 (Fig. 3). Thus, our results suggest that the nucleotide change in Hd17 results in down-regulation of the transcription of the floral repressor Ghd7, and that this is why 'Nipponbare' flowers earlier than those of NIL-Hd17.

The nucleotide change in Hd17 did not seem to affect Hd1 expression; however, its effect on flowering time disappeared in a background with a defective Hd1 allele, suggesting that Hd17 may regulate the Hd1 protein through a post-transcriptional mechanism (Fig. 4).

![Fig. 2](https://academic.oup.com/pcp/article-abstract/53/4/709/1841377) Comparison of transcription levels of three clock-related genes (OsLHY, OsPRR1 and OsGI) and two floral repressors (Hd1 and Ghd7) between 'Nipponbare' and NIL-Hd17 under long-day conditions. Values are means ± SD (n = 3). ZT, Zeitgeber time. Leaf samples were collected 20 d after germination.
Previously, Monna et al. (2002) mapped a flowering time QTL, referred to as $\text{Hd3b}$, on chromosome 6 using advanced backcrossed progeny between 'Nipponbare' and 'Kasalath' (an indica variety). $\text{Hd3b}$ was mapped within the same genomic region as $\text{Hd17}$, suggesting that $\text{Hd3b}$ and $\text{Hd17}$ may be at the same locus (Fig. 5A). Indeed, NIL-$\text{Hd3b}$, which contained the 'Kasalath'-type allele in the 'Nipponbare' background, flowered later than 'Nipponbare' (Monna et al. 2002), and the 'Kasalath' type allele has the same nucleotide change as 'Koshihikari' within the corresponding ORF (Fig. 5B, C). Furthermore, the transcription level of $\text{Ghd7}$ in 'Nipponbare' was significantly lower than that in NIL-$\text{Hd3b}$ at ZT 3, the same as the comparison with NIL-$\text{Hd17}$ (Fig. 5D). These pieces of genetic evidence support the hypothesis that $\text{Hd17}$ and $\text{Hd3b}$ are at the same locus.

To date, two genes that affect the transcription level of $\text{Ghd7}$ have been known. One is PHYTOCHROME B ($\text{PHYB}$; Osugi et al. 2010) and the other is $\text{Ehd3}$ (Matsubara et al. 2011). Both of these genes repress the $\text{Ghd7}$ mRNA level. These genes appear to be upstream of or in a pathway independent from that of $\text{Hd17}$, because $\text{PHYB}$ is a photoreceptor mediating light signals to the photoperiodic pathway, and there was no significant difference in the $\text{Ehd3}$ transcription levels between 'Nipponbare' and NIL-$\text{Hd17}$ (Supplementary Fig. S6). However, additional studies will be needed to assign their positions properly within the gene regulatory network of flowering in rice.

To examine the origin of the nucleotide change in $\text{Hd17}$, we surveyed the distribution of the SNP in Asian rice cultivars (both japonica and indica subspecies of $\text{O. sativa}$, $n = 73$) and their wild progenitors ($\text{O. rufipogon}$, $n = 37$) by means of sequencing (Fig. 6A). The SNP was observed among Asian rice cultivars, mainly in japonica. It seems that the wild-type allele has the 'Koshihikari' SNP, because almost all indica cultivars and wild accessions surveyed in this study carry the 'Koshihikari' SNP (Fig. 6A, Supplementary Table S2). This is supported by the fact that Arabidopsis ELF3 contains serine in the region that is highly conserved between the Arabidopsis and rice genomes (Supplementary Fig. S3). Given that the japonica cultivars originated from $\text{O. rufipogon}$ in China.
(Londo et al. 2006, Molina et al. 2011), the presence of the ‘Nipponbare’ SNP in Chinese japonica cultivars and in a wild accession implies that the SNP might have come from a Chinese japonica cultivar and that the footprint might be traceable to a wild progenitor from China, although we surveyed a limited number of accessions in our analysis and cannot prove this.

There was no obvious geographic pattern in the SNP among the Japanese japonica cultivars ($n = 57$) (Fig. 6B). This may be explained by the fact that cultivars grown in the northern region ($>37^\circ$C) often carry the deficient $Hd1$ allele (e.g. ‘Sasanishiki’) or the $Ghd7$ allele (e.g. ‘Hayamasari’) (Fujino et al. 2010, Matsubara et al. 2011, Shibaya et al. 2011), and by the fact that the nucleotide change only slightly modifies flowering under ND conditions.

Taken together, our results suggest that the ‘Nipponbare’ allele is a variant, and that the amino acid change (serine to leucine) that arose in the $Hd17$ protein may reduce the mRNA level of the floral repressor $Ghd7$, causing ‘Nipponbare’ to flower earlier than NIL-$Hd17$. On the other hand, a loss-of-function mutation $ef7$ in the rice $ELF3$-like gene seems to increase the $Ghd7$ transcription level, and the mutants flower later than the wild-type plants (Yuan et al. 2009, Saito et al. 2012), suggesting that the $ELF3$-like gene acts as a floral promoter by attenuating the $Ghd7$ transcription level, unlike Arabidopsis $ELF3$. These observations suggest that the amino acid change in $Hd17$ (‘Nipponbare’) accelerates rice flowering under ND and LD conditions.

In the present study, we found no obvious difference between ‘Nipponbare’ and NIL-$Hd17$ in the expression of clock-associated genes. However, this result does not rule out the possibility that $Hd17$ is involved in circadian rhythms. Because both alleles of $Hd17$ (‘Nipponbare’ and ‘Koshihikari’) were likely to be functional (Fig. 1E), the nucleotide change in $Hd17$ might not affect the expression of the clock-associated genes. Alternatively, it is possible that we failed to detect a difference in the abundances of their transcripts. To resolve these issues, further studies using a loss-of-function mutation in $Hd17$ should be conducted.

Recently, a QTL mapping study of natural variation in flowering time, which was accompanied by sequence analysis for the candidate genes, suggested that a large part of the variation in Asian rice cultivars could be explained by combinations of loss- and gain-of-function alleles of QTLs, rather than by QTLs with a small effect (Ebana et al. 2011). Generally speaking, this could explain large phenotypic differences between parents. In the present study, however, we demonstrated that a QTL with a small regulatory mutation (namely $Hd17$) also contributes to flowering time variation. Furthermore, previous fine mapping studies of a flowering-time QTL on chromosome 6 revealed that a single QTL was composed of multiple loci (including $Hd3b$/$Hd17$), and suggested that combinations of alleles at such loci are involved in the observed natural variation (Kojima et al. 2002, Monna et al. 2002, Hagiwara et al. 2009). With this in mind, further studies of the genetic bases and relationships with other genes in QTLs with a small effect...
Fig. 6 (A) Geographical distributions of the ‘Koshihikari’ and ‘Nipponbare’ SNP at the Hd17 locus in Asian rice cultivars (*O. sativa*) and their wild progenitors (*O. rufipogon*). Filled circles show the ‘Koshihikari’ SNP (C) and open circles show the ‘Nipponbare’ SNP (T), respectively. Details and additional data are shown in Supplementary Table S2. (B) Geographical distribution of the ‘Koshihikari’ and ‘Nipponbare’ SNPs at the Hd17 locus among the Japanese rice cultivars. Filled circles show the ‘Koshihikari’ SNP (C) and open circles show the ‘Nipponbare’ SNP (T).
will be necessary to understand fully the natural variation in rice flowering, even though this will be a difficult challenge.

**Materials and Methods**

**Plant materials and growth conditions**

NIL-Hd17 is a NIL derived from backcrossing (BC4) with 'Nipponbare', in which 'Koshihikari' was used as a donor of the Hd17 genomic region (Matsubara et al. 2008). Plants were grown in a controlled-environment cabinet (Espemic TGEH-9) under SD conditions (10 h light/14 h dark; 28°C for 12 h and 24°C for 12 h, respectively) or LD conditions (14.5 h light/9.5 h dark; 28°C for 12 h and 24°C for 12 h, respectively) at 60% relative humidity. Light was provided by metal halide lamps (300–1,000 nm spectrum, photosynthetic photon flux density of 500 μmol m⁻² s⁻¹). For evaluation under ND conditions, plants were also grown from mid-April in a paddy field in Tsukuba, Japan. The daylengths during vegetative growth were 13.1 h at germination (mid-April), 14.1 h at 30 d after germination (mid-May), 14.6 h at 60 d (mid-June), 14.4 h at 90 d (mid-July) and 13.5 h at 120 d (mid-August). Days to flowering under each condition were scored as the number of days required from germination to emergence of the first panicle.

**Map-based cloning of Hd17**

To test the complementation of Hd17, we cloned an approximately 9.4 kb genomic fragment of 'Koshihikari', which was digested by Xhol and BamHI and transformed into the pPZP2H-lac binary vector (Fuse et al. 2001). The resultant plasmid was then introduced into 'Nipponbare' by means of Agrobacterium-mediated transformation (Toki et al. 2006).

**Quantitative RT–PCR analysis of gene expression**

Quantitative reverse transcription-PCR (RT–PCR) analysis was performed as described previously (Ogiso et al. 2010). To quantify Hd17 transcription, we used the specific primers 5'-GCCCT CGCATCGCTTCA-3' and 5'-TTGAACGGCTGGAACAGA GAA-3' and the probe 5'-CCAGAGCCAGGGTATGGACGTG ACAG-3'. For transcription of other genes, we quantified the abundance in accordance with the methods in previously published papers (Ogiso et al. 2010 for OsLHY, OsPRR1 and OsGI; Matsubara et al. 2011 for Ghd7, Ehd1, Hd3a, RFT1 and UBQ2).

In addition, primers used in the fine mapping of Hd17 are described in Supplementary Table S3. RNA interference of Os06g0142650 and the bioluminescence assay for the circadian rhythm are described in Supplementary Text 1.

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


