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Research Paper

Genetic effect of a new allele for the flowering time locus *Ghd7* in rice

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The optimization of flowering time is a key aspect in maximizing grain productivity in rice. Allelic variations in genes for flowering time are major drivers in the wide adaptability of cultivated rice around the world. Here, we identified a novel allele of flowering time gene *Grain number, plant height and heading date 7* (*Ghd7*). Loss-of-function *ghd7*, *Ghd7*-0a, is important for extremely early flowering time for adaptability to cultivation in Hokkaido, Japan. However, the rice variety Sorachi lacks a key functional nucleotide polymorphism of *Ghd7*, which results in a loss of function of the gene. Based on the sequence of *Ghd7* allele in Sorachi, we identified the insertion of a transposon-like sequence at an upstream site of *Ghd7*. Segregation analysis using an F_2 population derived from the cross between Hoshinoyume and Sorachi demonstrated that the *Ghd7* locus contributed to extremely early flowering time in Sorachi. This *Ghd7* allele in Sorachi showed a weak function in terms of delay of flowering time, compared with loss-of-function allele, and a distinct distribution in northern Japan.

Key Words: flowering time, *Ghd7*, weak allele, rice.

Introduction

Flowering time in crop species is a major factor limiting species range and is a source of local adaptation. Variation in flowering time is determined by the response to seasonal cues from changing environmental factors including day length and temperature (Blümel *et al.* 2015, Hill and Li 2016, Hu *et al.* 2019). Allelic variation in genes for flowering time is a major driver of adaptability among cultivated species and their wild relatives.

Asian cultivated rice, *Oryza sativa* L., originated from the tropics. Extensive efforts by rice breeding programs to optimize flowering time have made rice production possible in various climatic conditions at latitudes ranging from 53°N to 40°S (Lu and Chang 1980). Early flowering time, through a decrease in photoperiod sensitivity, may have played an important role in expanding the range of rice (Izawa 2007, Shrestha *et al.* 2014, Zheng *et al.* 2016). Hokkaido (41–45°N latitude), is the northernmost region of Japan and one of the northern-limits of rice cultivation around the world (Fujino *et al.* 2019a). Rice requires extremely early heading to adapt to such cultivation conditions. Based on a series of genetic analyses for extremely early flowering time, we previously identified QTLs for extremely early flowering behavior using unique natural variations originating from Hokkaido.

Along with the human migration in Japan in the 1800s, *de novo* mutations causing loss-of-function in flowering time genes *Grain number, plant height and heading date 7* (*Ghd7*) and *Oryza sativa Pseudo-Response Regulator37* (*OsPRR37*) might be selected (Fujino *et al.* 2019a). *Ghd7* encodes a CO, CO-LIKE, and TIMING OF CAB1 (CCT) domain protein (Xue *et al.* 2008). *OsPRR37* is an ortholog of the circadian clock genes *PRR3/7* in *Arabidopsis* (Gao *et al.* 2014, Koo *et al.* 2013, Murakami *et al.* 2007, Nakamichi *et al.* 2005, Wei *et al.* 2010). Loss-of-function alleles caused by premature stop codons may generate extremely low photoperiod sensitivity, as seen in the population from Hokkaido (Fujino and Sekiguchi 2005a, 2005b, Fujino *et al.* 2019a, Tanisaka *et al.* 1992).

Previously, we identified genes responsible for the extremely early flowering time in rice varieties from Hokkaido (Fujino *et al.* 2019a, 2019b). However, 10 among 63 accessions in the Hokkaido Rice Core Panel (HRCP) do not carry the premature stop codon in the *Ghd7*-0a (Fujino *et al.* 2019a, Shinada *et al.* 2014, Xue *et al.* 2008). Here, we identified a new allele of *Ghd7* from these 10 accessions, and we characterized its structure and genetic effect on flowering time in rice.

Materials and Methods

Plant materials

Segregation analysis on flowering time in the F_2 and F_3

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generations of a cross between rice varieties Hoshinoyume (HS) and Sorachi (SR) were performed. SR is one of the 10 varieties without the premature stop codon among the HRCP, whereas HS carries the stop codon. SR carries a loss-of-function *hd1*, loss-of-function *dth8*, and unknown function *Ghd7(SR)*; *hd1dth8Ghd7(SR)*, whereas HS carries a functional *Hd1*, functional *DTH8*, and loss-of-function *ghd7*; *Hd1DTH8ghd7* (Fujino *et al.* 2019a). These materials were also used to investigate genetic interactions among *Hd1*, *DTH8*, and *Ghd7(SR)*. The F_2 population derived from self-pollination of the F_1 plants between them was used. Four F_2 plants heterozygous for *Hd1* were selected with all four combinations of *Ghd7* and *DTH8*. Four types of F_3 populations (96 plants/population), populations I–IV, were developed from self-pollination of the selected F_2 plants.

Seven populations of cultivars and landraces were used for the survey of the distribution of *Ghd7* allele types (**Supplemental Table 1**). Three populations, the HRCP, JRC, and HLP, had already been collected (Ebana *et al.* 2008, Fujino *et al.* 2019a, Shinada *et al.* 2014). Based on records of the varieties maintained in the Genebank in Japan, we collected four populations, VIB, Lthk, Bthk, and HKR, in this study.

Seeds of rice varieties were provided by the Genebank of NARO (Tsukuba, Japan) and the Local Independent Administrative Agency of Hokkaido Research Organization, Hokkaido Central Agricultural Experiment Station (Takikawa, Japan).

Full methods, including cultivation conditions, trait evaluation, and DNA analysis (**Supplemental Table 2**), are available in the **Supplemental Text 1**, and these were carried out using standard procedures as described previously. Sequence data from this article have been deposited in the EMBL/GenBank Databases under accession numbers LC472532 and LC472533.

Results

The 1901-bp sequence inserted into the Ghd7 gene

To elucidate the function of the *Ghd7* allele, we determined the DNA sequence of *Ghd7* in SR. There were three mutation events compared with *Ghd7*-2 in Nipponbare. A substitution in the 5′ upstream region and a 2-bp deletion in 3′ downstream region were observed (**Fig. 1A**). In addition, the single insertion of a 1901-bp sequence was detected (**Fig. 1A**).

This 1901-bp sequence was inserted at nucleotide position -12 bp relative to the transcription start site of *Ghd7*, Os07g0262100 in RAP-DB (https://rapdb.dna.affrc.go.jp/) (**Fig. 1B**). The inserted sequence displayed the structural features of a transposon: two long terminal repeats (LTRs), a single gene with a Zn-finger motif, and a gag-pol superfamily domain (**Fig. 1A**). In addition, a 5-bp target-site duplication (TSD) region was identified. A BLAST search (2019, 15, Feb) using the sequence as the query identified

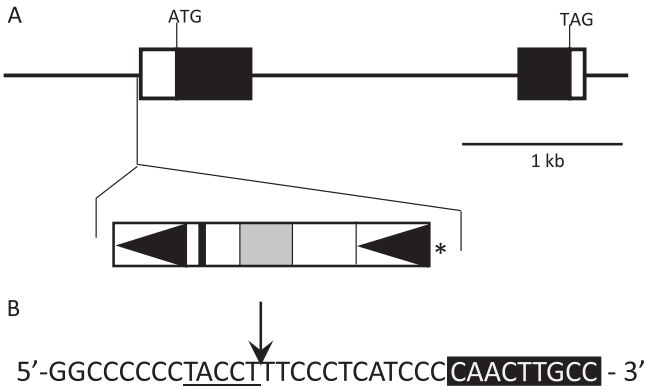

Fig. 1. Schematic representations of the *Ghd7* allele in Sorachi. A; Insertion into a position 12 bp upstream from the transcription start site of *Ghd7*, Os07g0262100 in RAP-DB (https://rapdb.dna.affrc.go.jp/). Open and closed boxes indicate the transcribed region and the coding sequence, respectively. The inserted sequence carries two direct repeats (closed triangle), a Zn-finger motif (closed box), and gag-pol superfamily domain (gray box). An asterisk indicates a 5-bp TSD, TACCT. B; The insertion site sequence. An arrow indicates the insertion site. Black and white letters indicate the upstream sequence and the transcribed region of *Ghd7*, respectively. Underlines indicate the TSD.

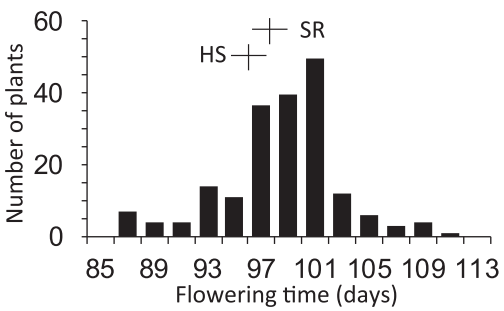

Fig. 2. Frequency distribution of flowering time in an F_2 population derived from a cross between Hoshinoyume (HS) and Sorachi (SR). Horizontal and vertical bars indicate range and mean of flowering time.

at least two sequences in the genome of the *japonica* and *indica* rice (**Supplemental Table 3**). The 1901-bp sequence inserted in the *Ghd7* allele in SR was named *Ghd7*-2tp.

Genetic effect of Ghd7-2tp on flowering time

To clarify an allele effect of *Ghd7*-2tp, we performed segregation analyses of flowering time in natural field conditions. At first, we used an F_2 population derived from the cross between HS and SR. The flowering times of the parents were 96.2 ± 1.0 days in HS and 98.3 ± 1.0 days in SR. Segregation of flowering time in the F_2 population (n = 193) was continuous (87.0–111.0 days) (**Fig. 2**). Significant differences in mean flowering time were detected at the two chromosomal regions corresponding to *Ghd7* and *DTH8* (**Table 1**). The flowering time of plants with *Ghd7*-2tp, 100.2 days, was significantly delayed compared with those with *Ghd7*-0a, 95.7 days.

Table 1. Flowering time in the F_2 population derived from the cross between Hoshinoyume (HS) and Sorachi (SR)

Marker	Chromosome	Targeted gene	Homozygous for the HS type			Homozygous for the SR type			Heterozygous		
			n	FT	Range	n	FT	Range	n	FT	Range
Hd1H43	6	*Hd1*	42	99.0 ± 6.2^{ns}	87–111	41	99.8 ± 2.2	93–101	82	97.9 ± 4.2	87–109
HD519del	8	*DTH8*	44	100.1 ± 3.0***	93–109	51	95.1 ± 4.1	87–103	73	100.2 ± 4.1	89–100
Ghd7c1	7	*Ghd7*	21	95.7 ± 4.7***	87–107	51	100.2 ± 4.0	91–111	83	98.8 ± 4.0	87–109

n indicates the number of plants. Flowering time (FT) is expressed as mean ± standard deviation (SD). Range (days) shows the variation of FT in each genotype.
Asterisks indicate significant differences in FT between plants with the chromosomal region including functional allele and the loss-of-function allele in the genes (*** $P < 0.001$, Student's *t* test).
ns; not significant by Student's *t* test.

Switching of the Hd1 genetic effect on flowering time by Ghd7-2tp

Next, to elucidate the genetic interactions of *Ghd7*-2tp with *Hd1* and *DTH8*, segregation of flowering time by the *Hd1* genotype was determined using all four combinations of *Ghd7* and *DTH8* as populations I to IV (**Fig. 3**). In population I, *Ghd7*-2tp*DTH8*, the flowering time of plants with functional *Hd1* was significantly delayed compared with those with loss-of-function *hd1* (**Fig. 3**, **Supplemental Table 4**). In contrast, the other three populations, populations II–IV, plants with *Hd1* significantly promoted flowering time compared with those with *hd1* (**Fig. 3**, **Supplemental Table 4**).

Ghd7-2tp might show the switching of the genetic effect of *Hd1* as the interactions of *Ghd7*-2tp with *Hd1* and *DTH8* (**Fig. 4**, **Supplemental Table 4**). *Hd1* promoted flowering time with *Ghd7*-2tp and loss-of-function *dth8*. Whereas, *Hd1* delayed flowering time with *Ghd7*-2tp and functional *DTH8*.

Distribution of Ghd7-2tp

To clarify the contribution of *Ghd7*-2tp to the extremely early flowering time, varieties of the HRCP were genotyped for the presence/absence of the insertion in the *Ghd7* gene (**Supplemental Table 5**). Nine of the 10 varieties without the premature stop codon included the insertion. The mean flowering time of the 53 rice varieties with *Ghd7*-0a was 85.7 ± 5.4 days (range 76.0–98.0 days; **Supplemental Table 6**). The mean flowering time of the nine varieties with *Ghd7*-2tp was 93.6 ± 4.2 days (range 89.2–99.2 days), which was a significant delay in flowering time compared with *Ghd7*-0a (**Supplemental Table 6**).

Next, to examine the evolutionary origin of *Ghd7*-2tp, we genotyped the *Ghd7* locus in a total of 367 varieties (**Table 2**). Among the JRC, only one variety, Fukoku, carried *Ghd7*-2tp (**Supplemental Table 7**). Only one variety, Kokoku, carried *Ghd7*-2tp among the HLP (**Supplemental Table 7**). Conversely, 14 and three varieties carried *Ghd7*-2tp among the Lthk and Bthk, respectively (**Supplemental Table 7**). No varieties carried *Ghd7*-2tp among the VIB and the HKR (**Supplemental Table 7**). The results

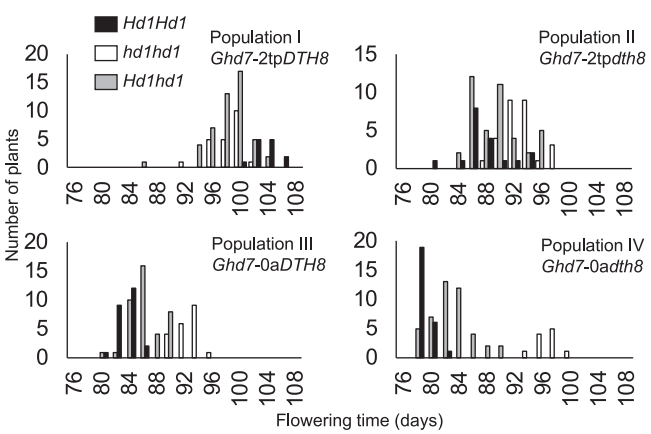

Fig. 3. Frequency distribution of flowering time in different genetic backgrounds with combinations of *Ghd7* and *DTH8*. Populations heterozygous for the *Hd1* gene had all four genetic combinations of *Ghd7* and *DTH8*. Population I, *Ghd7*-2tp*DTH8*, population II, *Ghd7*-2tp*dth8*, population III, *Ghd7*-0a*DTH8*, population IV, *Ghd7*-0a*dth8*. Black, white, and gray bars indicate the three genotypes of the *Hd1* gene; *Hd1*/*Hd1*, *hd1*/*hd1*, and *Hd1*/*hd1*, respectively.

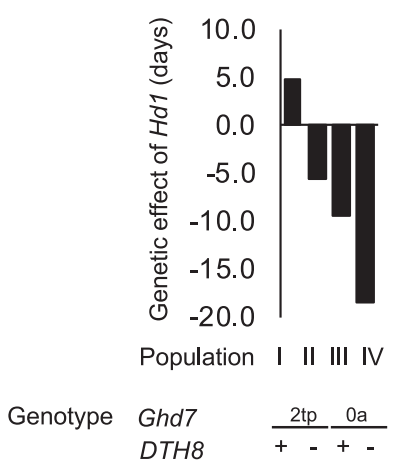

Fig. 4. Genetic effects of *Hd1* on flowering time in different genetic backgrounds of *Ghd7* and *DTH8* expressed as a difference in flowering time between plants carrying *Hd1Hd1* and *hd1hd1*. 2tp and 0a indicate the *Ghd7* alleles, which are functional and loss-of-function, respectively. + and – indicate functional and loss-of-function alleles of *DTH8*, respectively.

Table 2. Distribution of *Ghd7*-2tp allele in Japan

Population		The *Ghd7* allele	
Name	Size (n)	*Ghd7*-2tp	WT
JRC	48	1	47
VIB	21	0	21
HRCP	63	9	54
HLP	43	1	42
Lthk	152	14	138
Bthk	29	3	26
HKR	11	0	11

Number of varieties with each allele is shown. Population is described in **Supplemental Table 1**.
Ghd7-2tp carry the insertion. WT indicate no insertion at the site.

suggested that *Ghd7*-2tp distributed in the small number of varieties from the northern Japan depending on their genetic effect on flowering time.

Discussion

The optimization of flowering time is a key aspect of grain productivity in rice. *Ghd7* may contribute to the adaptability in rice (Fujino and Sekiguchi 2005a, Fujino *et al.* 2019a, 2019b, Xue *et al.* 2008). Compared with loss-of-function *Ghd7* allele, *Ghd7*-0a, the new allele of *Ghd7*, *Ghd7*-2tp, identified in this study showed an altered genetic effect on flowering time (**Fig. 2**, **Table 1**).

The 1901-bp sequence inserted into *Ghd7* was identical to that of *Hd1* in Taichu No. 65 (Doi *et al.* 2004). The 1901-bp sequence had the structural features of a transposon and were present in both *indica* and *japonica* genomes (**Fig. 1**, **Supplemental Table 3**). *Ghd7*-2tp was distinctly distributed in varieties from northern Japan (**Table 2**). No varieties grown in central-southern Japan carried the *Ghd7*-2tp allele. In addition, *Ghd7*-2tp was not detected in cultivated rice collected from around the world (Lu *et al.* 2012, Xue *et al.* 2008). The results suggested that insertion of the transposon-like is most likely a *de novo* mutation that occurred after rice cultivation became stable as agriculture in the Tohoku region of northern Japan.

Allelic variation in genes for flowering time is a major driver of adaptability among cultivated species and their wild relatives, which are generated from *de novo* mutations and natural selection. Transposons play a major role in the generation of allelic variation, altering gene expression, and driving genome evolution (Dubin *et al.* 2018, Zhao *et al.* 2016). Transposons are the largest component of various eukaryote genomes including rice (International Rice Genome Sequencing Project 2005). Therefore, it is unclear what the actual role of transposon insertions in genes may have on the regulation of flowering time. *Ghd7*-2tp identified in this study may shed light on the role of transposons on not only evolutional studies but also plant breeding programs.

We previously elucidated that the dual function of *Hd1* is regulated by *Ghd7* and *OsPRR37* (Fujino *et al.* 2019b, Hayama *et al.* 2003, Yano *et al.* 2000). Functional *Ghd7* and *OsPRR37* can switch the genetic effects of *Hd1* from the promotion to the delay of flowering times (Fujino *et al.* 2019b). The genetic effect of *Hd1* can be switched by *Ghd7*-2tp in conditions with *DTH8* (**Fig. 4**, **Supplemental Fig. 1**). Our results further enhance the molecular dissection of flowering time control in rice.

Note added in proof

While this paper was under review, a new allele of *Ghd7*, which is the same as *Ghd7*-2tp, was described (Saito *et al.* 2019).

Author Contribution Statement

Conceived and designed the experiments and wrote the manuscript: KF. Performed the experiments, analyzed the data, and approved the final manuscript: KF and UY.

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