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ORIGINAL PAPER

Genetic interactions involved in the inhibition of heading by heading date QTL, *Hd2* in rice under long-day conditions

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Abstract Heading date is the one of the most important traits in rice breeding, because it defines where rice can be cultivated and influences the expression of various agronomic traits. To examine the inhibition of heading by *Heading date 2* (*Hd2*), previously detected on the distal end of chromosome 7's long arm by quantitative trait locus (QTL) analysis, we developed backcross inbred lines (BILs) from Koshihikari, a leading Japanese cultivar, and Hayamasari, an extremely early heading cultivar. The BILs were cultivated under natural field conditions in Tsukuba Japan, and under long-day (14.5 h), extremely long-day (18 h), and short-day (10 h) conditions. Combinations of several QTLs near *Hd1*, *Hd2*, *Ghd7*, *Hd5*, and *Hd16* were detected under these four conditions. Analysis of advanced backcross progenies revealed genetic interactions between *Hd2* and *Hd16* and between *Hd2* and *Ghd7*. In the homozygous Koshihikari genetic background at *Hd16*, inhibition of heading by the Koshihikari allele at *Hd2* was smaller than that with the Hayamasari *Hd16* allele. Similarly, in the homozygous Koshihikari genetic background at *Ghd7*, the difference in heading date caused by different alleles at *Hd2* was smaller than in plants homozygous for the Hayamasari *Ghd7* allele. Based on these results, we conclude that *Hd2* and its genetic interactions play an important role in controlling heading under long-day conditions. In addition, QTLs near *Hd2*, *Hd16*, and *Ghd7*, which are involved in inhibition of heading under long-day conditions, function in the same pathway that controls heading date.

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Introduction

Rice heading date (flowering time) is one of the most important traits in breeding programs, because it influences the expression of various agronomic traits and the regions where rice can be grown (i.e., the seasonal adaptation of rice cultivars). To modify heading date in a breeding program, it is necessary to understand the genetic factors and genetic control mechanisms involved in this process. To this end, many molecular and genetic analyses have been performed on rice heading date (Izawa 2007; Izawa et al. 2003; Yano et al. 2001). In particular, quantitative trait locus (QTL) analysis has contributed significantly to our understanding of the genetic control of heading date in rice. Many QTLs have been identified and some have also been cloned using a map-based strategy (Izawa 2007; Xue et al. 2008). Moreover, Ebana et al. (2011) have recently demonstrated that naturally occurring variation in heading date among diverse Asian germplasms could be generated by combination of gain- and loss-of function alleles at the previously identified loci *Hd1*, *Hd2*, *Hd5*, *Hd6*, *RFT1*, *Ghd7*, and *Hd16*. However, although interactions among these genes have been partially revealed by molecular cloning (Izawa 2007; Xue et al. 2008), our image of the genetic

control network for heading date in rice remains incomplete.

Heading date 2 (*Hd2*), one of the QTLs for heading date, has been previously detected at the distal end of the long arm of chromosome 7 in an F_2 population derived from a cross of Nipponbare, a *japonica* variety, and Kasalath, an *indica* variety (Yamamoto et al. 1998; Yano et al. 1997) and was also detected in backcross inbred lines (BILs) that used Nipponbare as the recurrent parent (Lin et al. 1998). On the other hand, *Hd2* has not been detected in backcross progeny and chromosome segment substitution lines derived from a cross between Koshihikari and Kasalath (Ebitani et al. 2005; Yamamoto et al. 2001). These results have suggested that Nipponbare and Koshihikari might have different *Hd2* alleles. However, in a set of reciprocal BILs from crosses between Nipponbare and Koshihikari, QTL analysis of heading date detected QTLs only on chromosomes 3 and 6, and no QTL was detected on chromosome 7 (Matsubara et al. 2008), suggesting that Nipponbare and Koshihikari may have the same *Hd2* allele. These inconsistent detections of *Hd2* in several cross combinations and generations suggest that a genetic interaction may be involved in the ability of *Hd2* to delay heading.

Hayamasari is a *japonica* cultivar that is adapted to growing in the northernmost region of Japan (i.e., Hokkaido), and shows both extremely early heading and no response to day-length (Fujino and Sekiguchi 2005a; Nonoue et al. 2008). It has been previously suggested that Hayamasari has an *Hd2* allele that decreases the number of days to heading (DTH) based on the genetic analysis of BILs derived from Hayamasari and Italica Livorno (Fujino and Sekiguchi 2005a), indicating that Hayamasari's *Hd2* allele is unique to adapt the northernmost region of Japan and that contributes to expansion of rice cultivation area to the north.

In the present study, we set out to clarify the previous contradictory results for *Hd2* by developing Koshihikari × Hayamasari BILs and performing QTL analysis for heading date under different environments: natural field (NF), long day (LD: 14.5 h light), extremely long day (ELD: 18 h light), and short day (SD: 10 h light). We successfully detected a QTL at the distal end of the long arm of chromosome 7, which corresponds to *Hd2*. We also developed advanced backcross progeny to examine genetic interactions involved in the inhibition of heading between *Hd2* and two other QTLs, which correspond to *Hd16* (Matsubara et al. 2008) and *Ghd7* (Xue et al. 2008). Our results strongly supported the hypothesis that *Hd2* plays an important role in the inhibition of heading under long-day conditions. We also clearly demonstrated that genetic interactions with *Ghd7* and *Hd16* are involved in the inhibition of heading by *Hd2*.

Materials and methods

Plant materials

We used two Japanese *japonica* cultivars, Koshihikari and Hayamasari, in this study. To develop the BIL population, we crossed Koshihikari with Hayamasari. The resultant F_1 plants were backcrossed with Koshihikari to obtain BC_1F_1 plants. The BC_1F_1 plants were consecutively self-pollinated four times using the single-seed-descent method to obtain BC_1F_5 plants. For our QTL analysis of heading date, we used 93 BC_1F_5 plants for the genotyping using DNA markers. We self-pollinated these 93 plants to produce 93 BC_1F_6 lines, which were planted and scored for heading date. All heading dates were scored as DTH.

To further confirm the effect of any QTL alleles that we detected and to examine their interactions, we developed advanced backcross progeny. An F_1 population of Koshihikari × Hayamasari was consecutively backcrossed four times with Koshihikari as the recurrent parent, and DNA markers were employed to select two BC_4F_1 plants in which the target QTL regions had been heterozygous for the Koshihikari genetic background in each preceding generation. The progenies of two self-pollinated BC_4F_1 plants (i.e., BC_4F_2) were used to examine genetic interactions between the QTLs that we detected.

Cultivation and scoring of DTH

We cultivated the BILs (BC_1F_6) that we developed and the advanced backcross progenies (BC_4F_2) in a paddy field at the National Institute of Agrobiological Sciences (NIAS) in Tsukuba, Japan, from April to September. The mean day-length during the cultivation period was 13.1 h in mid-April, 14.1 h in May, 14.6 h in June, 14.4 h in July, 13.5 h in August, and 12.4 h in September. Average temperatures during the cultivation period were 17°C in May, 22°C in June, 27°C in July, 28°C in August, and 24°C in September. Cultivation management followed the standard procedures used at NIAS. In the BILs, we scored the DTH of the first panicle for five plants per line and used the mean value for our QTL analysis. In the backcross progeny, DTH was scored for each plant using 171 BC_4F_2 plants for the interaction between *Hd2* and *Hd16* and using 331 BC_4F_2 plants for the interaction between *Hd2* and *Ghd7* in the field. We also grew the BILs and the advanced backcross progeny used to test for interactions between *Hd2* and *Hd16* (92 plants) under ELD conditions (18 h light, with 28°C for 12 h and 24°C for 12 h), and grew the BILs under LD conditions (14.5 h light, with 28°C for 12 h and 24°C for 12 h) and SD conditions (10 h light, with 28°C for 12 h and 24°C for 12 h) in a TGEH-9 controlled-growth cabinet (ESPEC

MIC Corp, Tokyo, Japan). In the cabinet, DTH was scored for three plants per line in the BILs and for each plant in the advanced backcross progeny for the test of interaction between *Hd2* and *Hd16*.

DNA marker analysis

We extracted total DNA from the leaves of the BC_1F_5 plants, advanced backcross progenies, and parental lines using the CTAB method (Murray and Thompson 1980). In the BILs, we mainly used single-nucleotide polymorphism (SNP) markers and a few simple sequence repeat (SSR) markers. In the advanced backcross progenies, we used SSR markers. The 372 SNPs used for genotyping were selected from genome-wide SNP marker data (Nagasaki et al. 2010; Yamamoto et al. 2010). The SSR markers were selected from those reported by McCouch et al. (2002). All markers used in the BILs are listed in Supplemental Table S1. The SNPs were detected using the BeadStation 500G system (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. For the polymerase chain reaction (PCR) of the SSR markers, we used a 10-μL reaction volume containing 1 μL of template DNA (20 ng/μL), 5 μL of 2 × Go-Taq Green Master Mix (Promega, Fitchburg, WI, USA), 0.2 μL of each primer solution (20 pmol/μL), and 3.6 μL H_2O. Amplification was performed for 35 cycles (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C), followed by 7 min of final extension at 72°C. The amplified products were separated by electrophoresis in a 3% agarose gel to detect polymorphisms.

Linkage map and QTL analysis

We constructed the linkage map using MAPMAKER/EXP 3.0 (Lander et al. 1987), with the Kosambi function used to calculate genetic distances (in cM). In the BILs, we treated the genotype of the residual heterozygous region as missing data. We performed the QTL analyses by means of composite interval mapping (Zeng 1993, 1994), as implemented by the Zmapqtl module (model 6) provided by version 2.5 of the QTL Cartographer software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm; see also Basten et al. 2005). We used genome-wide threshold values ($\alpha = 0.05$) to detect putative QTLs based on the results of 1,000 permutations (Churchill and Doerge 1994).

Evaluation of genetic interactions

To analyze genetic interactions among the QTLs we detected, we classified the BC_4F_2 plants into nine classes according to the genotypes of SSR markers tightly linked to three QTLs (RM1357 for the QTL at the distal end of chromosome 7 assumed to be *Hd2*, RM1038 for the QTL on the long arm of chromosome 3 assumed to be *Hd16*, and RM21327 for the QTL in the proximal region of chromosome 7 assumed to be *Ghd7*). We compared the mean DTH between the nine genotypes using two-way analysis of variance (ANOVA) for the two BC_4F_2 populations to test for an additive effect of the allele of the three target QTLs. Testing for each genetic interaction types (AA, additive by additive; AD, additive by dominance; DA, dominance by additive; DD, dominance by dominance) was done by orthogonal contrast test (Kao and Zeng 2002).

Results

Heading behavior of the parental lines under four environmental conditions

We scored DTH of the parental lines (Koshihikari and Hayamasari) under NF conditions at Tsukuba, and under ELD, LD, and SD conditions in the growth cabinet. To examine photoperiod sensitivity, comparison of DTH under ELD, LD, and SD conditions in the growth cabinet is meaningful because we need not consider other factors, such as temperature and growth conditions affecting on DTH. In the growth cabinet, DTH of Koshihikari increased with increasing day-length, thereby showing a photoperiod sensitivity (Fig. 1). On the other hand, Hayamasari had earlier heading under ELD and LD conditions than under SD conditions, indicating that it shows no photoperiod sensitivity, as was reported previously (Nonoue et al. 2008). The DTH of Hayamasari was almost the same under EDL and LD conditions. Under SD conditions, the DTH of Koshihikari and Hayamasari was almost the same, but under ELD conditions, Hayamasari headed 68 days earlier than Koshihikari because of their different photoperiod sensitivities. Under NF conditions, Hayamasari headed about 30 days earlier than Koshihikari. In both parent, the DTH was significantly increased under NF conditions than LD conditions, although a pattern of day-length response in NF conditions was like that in LD conditions. The differences in DTH between these two conditions were probably caused by low temperature in May (17°C at Tsukuba, Japan) in NF conditions and different cultivation practice (transplanting in NF, but not in LD).

Variations in DTH of the BILs under four environmental conditions

The BILs generally showed continuous variation in DTH, ranging from 75 to 125 days under NF conditions (Fig. 2a), 50–71 days under SD conditions (Fig. 2b), and 50–110 days under LD conditions (Fig. 2c). Under ELD conditions, the BILs showed a wide range of DTH

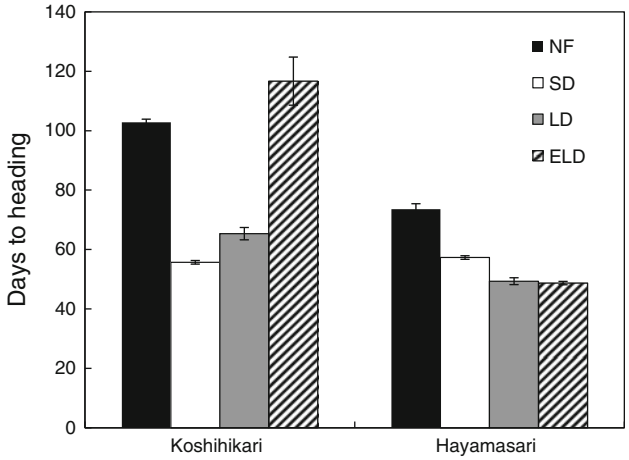

Fig. 1 Days to heading of the parental lines Koshihikari and Hayamasari under natural field (NF), short-day (SD: 10 h light), long-day (LD: 14.5 h light), and extremely long-day (ELD: 18 h light) conditions. The data represent mean values ($n = 3$ plants for SD, LD, and ELD; $n = 5$ for NF) ± standard deviations

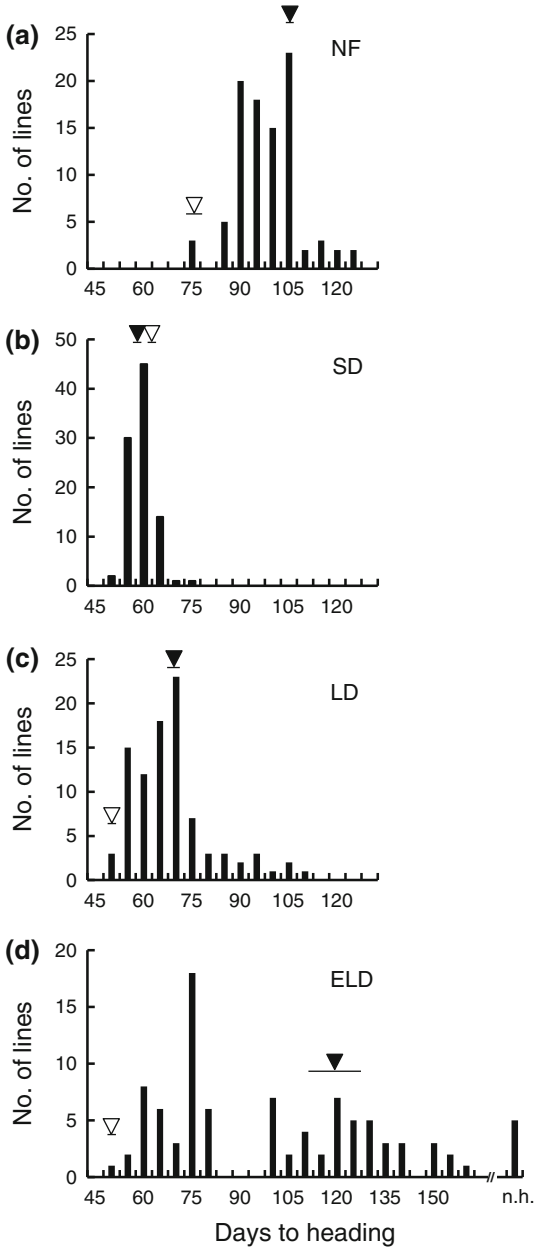

Fig. 2 Frequency distributions for the number of days to heading (DTH) of the Koshihikari × Hayamasari BILs under **a** natural field (NF), **b** short-day (SD: 10 h light), **c** long-day (LD: 14.5 h light), and **d** extremely long day (ELD: 18 h light) conditions. *Black and white arrowheads* indicate the mean DTH of Koshihikari and Hayamasari, respectively; *horizontal lines* under the *arrowheads* indicate standard deviations (*n.h.* no heading after 188 days)

(Fig. 2d), from 50 to no heading after 188 days, but were divided into three groups: early heading (50–80 days), late heading (100–160 days), and no heading (after 188 days). Transgressive segregation was observed toward late heading under NF, ELD, and LD conditions.

QTL analysis of the BILs under four environmental conditions

Supplemental Figure S1 shows the linkage maps of the BILs for the marker names (abbreviations) listed in Supplemental Table S1. Table 1 summarizes the QTLs detected in the BILs under the four conditions, and Fig. 3 shows their positions. Under NF conditions, we detected four QTLs: one on chromosome 3 (near SNP marker NIAS_Os_aa03002556), two on chromosome 7 (near NIAS_Os_ab07000450 and NIAS_Os_aa07007522), and one on chromosome 8 (SSR marker RM4085). The Koshihikari allele decreased DTH at the QTL detected on chromosome 3, but increased DTH in the other QTLs we detected. Under LD conditions, we detected four QTLs: one on chromosome 3 (NIAS_Os_aa03002556), one on chromosome 6 (NIAS_Os_ab06001076), one on chromosome 7 (NIAS_Os_ab07000450), and one on chromosome 8 (RM4085). The Koshihikari allele decreased DTH for the QTLs we detected on chromosomes 3 and 6, and increased DTH in the other QTLs. Under ELD conditions, we detected four QTLs: one on chromosome 6 (NIAS_Os_ab06001076), two on chromosome 7 (NIAS_Os_ab07000450 and NIAS_Os_aa07001432), and one on chromosome 8 (RM4085). The Koshihikari allele decreased DTH for the QTL detected on chromosome 6, and increased DTH in the other QTLs. Under SD conditions, we detected two QTLs: one on chromosome 3 (NIAS_Os_aa03002705) and one on chromosome 6 (NIAS_Os_ab06000799). The Koshihikari allele increased DTH in the QTL on chromosome 3, and decreased DTH in the other QTL.

Table 1 Heading date QTLs detected in the Koshihikari × Hayamasari backcross inbred lines (BILs) under natural field (NF), long-day (LD: 14.5 h), extremely long-day (ELD: 18 h), and short-day (SD: 10 h) conditions

Environmental conditions	Nearest marker	Chr	LOD[a]	Additive effect[b]	PVE[c] (%)	QTL[d]
NF	NIAS_Os_aa03002556	3	4.84	−3.32	7.4	*Hd16*
	NIAS_Os_ab07000450	7	23.16	7.69	43.2	*Ghd7*
	NIAS_Os_aa07007522	7	6.62	3.13	7.9	*Hd2*
	RM4085	8	13.18	4.41	18.2	*DTH8* (*Hd5*, *Ghd8*)
LD	NIAS_Os_aa03002556	3	10.22	−7.37	20.1	*Hd16*
	NIAS_Os_ab06001076	6	3.78	−4.42	5.8	
	NIAS_Os_ab07000450	7	11.49	7.09	21.9	*Ghd7*
	RM4085	8	9.99	5.77	18.7	*DTH8* (*Hd5*, *Ghd8*)
ELD	NIAS_Os_ab06001076	6	4.70	−16.00	7.9	
	NIAS_Os_ab07000450	7	12.98	25.66	27.9	*Ghd7*
	NIAS_Os_aa07001432	7	6.45	15.89	11.9	*Hd2*
	RM4085	8	13.47	23.34	29.6	*DTH8* (*Hd5*, *Ghd8*)
SD	NIAS_Os_aa03002705	3	6.64	2.02	18.7	*Hd16*
	NIAS_Os_ab06000799	6	7.10	−2.15	25.7	*Hd1*

All genetic parameters were calculated by means of composite interval mapping using QTL Cartographer ver. 2.5 (Basten et al. 2005)

Chr chromosome number

[a] Log-likelihood value

[b] Additive effect of the Koshihikari allele on days to heading (DTH)

[c] Percent of phenotypic variance explained by the QTL

[d] Previously identified QTLs corresponding to the QTLs detected in this study based on their physical positions

Relationships between the QTLs detected in this study and those in previous reports

It is possible to predict the relationships between the QTLs detected in this study and those that have been previously reported based on their chromosomal locations. The genomic region of the QTL detected on chromosome 7 near NIAS_Os_ab07000450 appears to correspond to that of *Ghd7* (Fig. 3), which was previously identified as a heading-date QTL (Xue et al. 2008). Matsubara et al. (2011) reported that Hayamasari *Ghd7* was a loss-of-function allele based on its sequence (GenBank accession number AB517628.1), and Ebana et al. (2011) reported that Koshihikari *Ghd7* was a functional allele based on its sequence. Therefore, we believe that the QTL that we detected was *Ghd7*. Similarly, the QTL on chromosome 8 near RM4085 (Fig. 3) appears to correspond to *DTH8* (Wei et al. 2010). *DTH8* has been cloned and is considered to encode the nuclear transcription factor Y beta subunit (NF-YB). Sequence analysis of *DTH8* revealed that a 19-bp deletion has occurred in the Hayamasari *DTH8* allele, and that the sequence is clearly different from that of Koshihikari (GenBank accession number AB124652.1). These results suggest that the QTL we detected on chromosome 8 corresponds to *DTH8*. *Hd5*, which has been previously detected in QTL analysis of the progeny derived from Nipponbare and Kasalath, is likely to be *DTH8* based on a comparison between the chromosomal positions of the two QTLs (Lin et al. 2003; Yamanouchi et al., unpublished data; Yano et al. 1997).

In previous reports, *Hd2* has been mapped at the distal end of the long arm of chromosome 7 (Yamamoto et al. 1998; Yano et al. 1997), and its chromosomal position corresponded well to that of the QTL we detected on distal end of chromosome 7. Although we cannot rule out the possibility that two tightly linked QTLs are found at that locus, it appears likely that the QTL we detected in this study is *Hd2*. Based on the abovementioned evidence, we have concluded the QTLs that we detected on chromosome 7 near NIAS_Os_ab07000450 as *Ghd7*, the QTL near the distal end of chromosome 7 at NIAS_Os_aa07007522 as *Hd2*, and the QTL on chromosome 8 near RM4085 as *DTH8* (*Hd5*).

Two QTLs related to heading date, *Hd6* (Takahashi et al. 2001) and *Hd16* (Matsubara et al. 2008), were previously reported to lie at the distal end of chromosome 3. Previous sequence analysis has shown that Koshihikari has a loss-of-function allele at *Hd6*, which can be identified by a SNP (Matsubara et al. 2008; Takahashi et al. 2001). The *Hd6* allele of Hayamasari was examined using a cleaved amplified polymorphic sequence (CAPS) marker that identifies the functional SNP in *Hd6*, suggesting that Hayamasari has

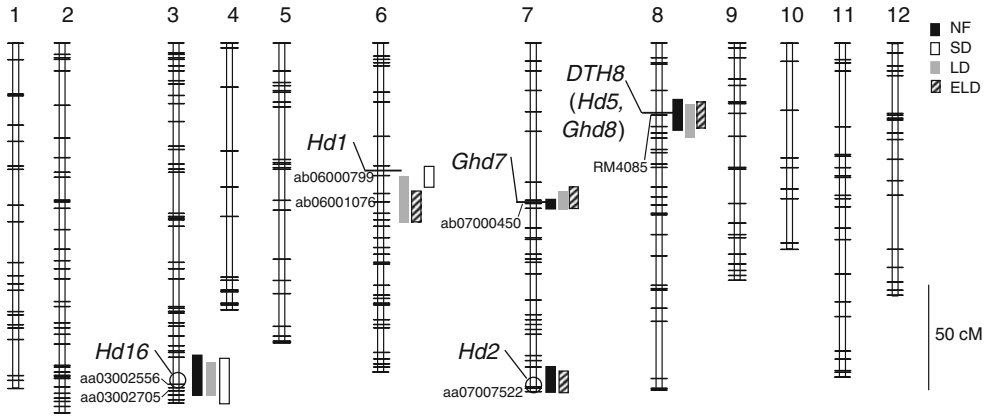

Fig. 3 Chromosomal locations of the heading date QTLs detected in the Koshihikari × Hayamasari BILs under natural field (NF), long-day (LD: 14.5 h), extremely long-day (ELD: 18 h), and short-day (SD: 10 h) conditions. The lengths of the *rectangles* indicate a two-LOD confidence interval for the QTLs. The *small horizontal bars* and *small letters* show the positions of the markers and the names of the marker nearest to the LOD peak, respectively. The *thick horizontal lines* and the *circles* with *italicized names* represent the positions of the QTLs for heading date identified in previous studies (Lin et al. 2003; Matsubara et al. 2008; Wei et al. 2010; Yamamoto et al. 1998; Yan et al. 2011; Yano et al. 2000). All SNP and SSR markers used for our analysis of the BILs are listed in Supplemental Table S1

the same loss-of-function allele as Koshihikari (data not shown). Therefore, the QTL detected under NF and LD conditions appears to be *Hd16* based on its chromosomal position (Matsubara et al. 2008). Under SD conditions, we detected a QTL on chromosome 6, and its position was corresponded to the *Hd1*, identified previously (Yano et al. 2000). Sequence analysis has revealed that Hayamasari has a functional *Hd1* allele different from that of Koshihikari (Nonoue et al. 2008). Therefore, the QTL detected on chromosome 6 under SD conditions appears to be *Hd1*. On the other hand, under LD and ELD conditions, the chromosomal positions of detected QTLs on chromosome 6 were near *Hd1* but not exactly corresponded to the position of previously reported *Hd1* (Fig. 3; Table 1).

Genetic interactions between *Hd2* and *Hd16*

Figure 4a shows the graphical genotype of the BC_4F_1 plants. To examine the genetic interactions between *Hd2* and *Hd16*, we scored heading dates in the BC_4F_2 population under NF conditions (Fig. 4b, d) and ELD conditions (Fig. 4c, e). In this population, five chromosomal regions including the *Hd2* and *Hd16* regions (grey regions in Fig. 4a) were segregating. Under NF conditions, the BC_4F_2 population showed continuous variation in DTH, ranging from 98 to 115 days (Fig. 4b). QTL analysis of the heading date confirmed the association between DTH and the marker genotypes only in the *Hd2* and *Hd16* regions and not in the other three chromosomal regions (Supplemental Table S2). The BC_4F_2 plants were classified into nine genotype classes based on the *Hd2* and *Hd16* alleles, and we compared the average DTH among the nine classes. Two-way ANOVA clearly indicated that the allele effects of *Hd2* and *Hd16* were not additive under NF conditions ($P < 0.0001$). Orthogonal contrast test showed that AA ($P < 0.01$) types of interaction was significant in the interaction of *Hd2* and *Hd16* under NF conditions. The effect of allelic substitutions at the *Hd2* locus differed depending on the *Hd16* genotype (Fig. 4d). In plants homozygous for the Hayamasari *Hd16* allele, homozygotes for the Koshihikari *Hd2* allele were later heading by about 14 days than homozygotes for the Hayamasari *Hd2* allele; in plants homozygous for the Koshihikari *Hd16* allele, the difference in DTH between the genotypes at *Hd2* was only about 3 days. On the other hand, in plants homozygous for the Koshihikari *Hd2* allele, homozygotes for the Hayamasari *Hd16* allele were later heading by about 12 days than homozygotes for the Koshihikari *Hd16* allele; in plants homozygous for the Hayamasari *Hd2* allele, the difference in DTH between the genotypes at *Hd16* was not completely observed.

Similar genetic interactions were observed under ELD conditions. The BC_4F_2 population showed large variations in DTH, ranging from 83 days to no heading observed even after 188 days, with three peaks (Fig. 4c). QTL analysis for DTH in this population revealed that *Hd2* was detected, but *Hd16* was not (Supplemental Table S2). Although QTL analysis did not detect *Hd16*, ANOVA revealed a significant interaction between *Hd2* and *Hd16* under ELD conditions ($P = 0.0008$). Furthermore, orthogonal contrast test showed that AA ($P < 0.01$) and DA ($P = 0.018$) types of interaction were significant in the interaction of *Hd2* and *Hd16* under ELD conditions. The effect of the Koshihikari *Hd2* allele on heading inhibition was clearly observed in the genotype with the Koshihikari *Hd16* allele under ELD conditions (about 35 days), versus little effect (about 3 days) under NF conditions (Fig. 4d, e). This indicates that

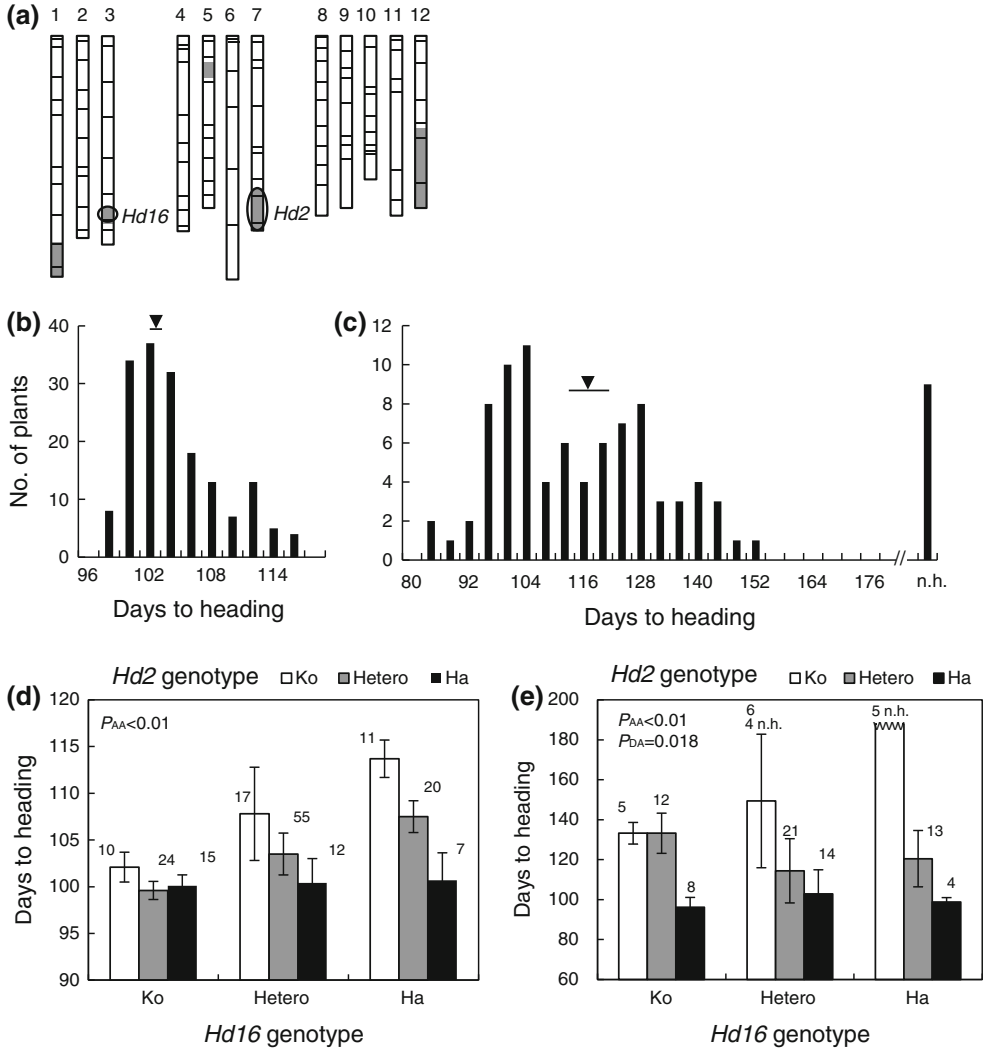

Fig. 4 Genetic interactions between *Hd2* and *Hd16* in the BC_4F_2 advanced backcross progenies. **a** Graphical genotypes of the selected BC_4F_1 plants. *White and grey regions* represent homozygous for Koshihikari and heterozygous, respectively. *Circles* show the position of the target QTLs. *Horizontal lines* indicate the positions of the SSR markers. Frequency distributions of days to heading under **b** NF conditions (171 plants) and **c** ELD (18 h) conditions (92 plants) in the BC_4F_2 population. (*n.h.* no heading after 188 days). *Black arrowheads* and *horizontal lines* under them indicate the mean DTH of Koshihikari and standard deviations, respectively. The mean DTH of Hayamasari under NF condition and under ELD conditions were 73.6 ± 1.8 and 48.7 ± 0.6 (mean ± standard deviations), respectively. Differences between *Hd2* and *Hd16* in days to heading for nine genotype classes under **d** NF conditions and **e** ELD conditions in the BC_4F_2 population. Genotypes were determined using the closely linked markers RM1357 (*Hd2*) and RM1038 (*Hd16*). Ko, Hetero, and Ha indicate homozygous for the Koshihikari allele, heterozygous, and homozygous for the Hayamasari allele, respectively. *Numerals* above the *bars* indicate the number of plants in each genotype class. Bars are mean values ± standard deviations. P_{AA} and P_{DA} are significant probabilities for AA and DA interactions between *Hd2* and *Hd16*, respectively. Under ELD conditions, in the class with the Koshihikari allele at *Hd2* and heterozygous alleles at *Hd16* (**e**), four plants did not head and the other six plants headed. In this class, mean value was calculated by assuming 188 days to heading for the plants that did not head

the effect of *Hd2* did not depend completely on the *Hd16* allele. On the other hand, the effect of the *Hd16* allele depended completely on having the Koshihikari *Hd2* allele, as was observed under NF conditions. Interestingly, none of the plants homozygous for both the Koshihikari *Hd2* allele and the Hayamasari *Hd16* allele headed under ELD conditions even after 188 days of cultivation, and four of ten plants homozygous for the Koshihikari *Hd2* allele and heterozygous for the Hayamasari *Hd16* allele also did not head. These results suggest that *Hd2* accompanying *Hd16* had a critical effect on rice heading under ELD conditions.

Genetic interactions between *Hd2* and *Ghd7*

We examined the interaction between *Hd2* and *Ghd7* in the BC_4F_2 population under NF conditions (Fig. 5). Figure 5a shows the graphical genotype of the BC_4F_1 plants that we used. The BC_4F_2 population showed a wide range of

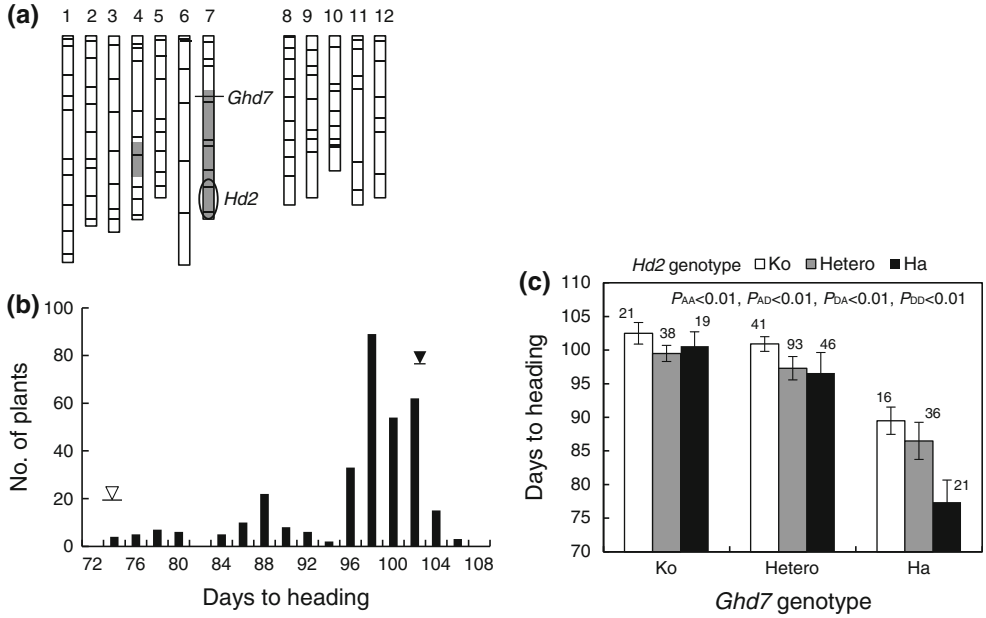

Fig. 5 Genetic interactions between *Hd2* and *Ghd7* in the BC_4F_2 advanced backcross progenies. **a** Graphical genotypes of the selected BC_4F_1 plants. *White and grey regions* represent homozygous for Koshihikari and heterozygous, respectively. *Circle* and *horizontal lines* indicate the positions of the target QTLs. *Horizontal lines without letters* indicate the positions of the SSR markers. **b** Frequency distributions for days to heading under natural field (NF) conditions (331 plants) in the BC_4F_2 population. *Black and white arrowheads* indicate the mean DTH of Koshihikari and Hayamasari, respectively; *horizontal lines* under the *arrowheads* indicate standard deviations. **c** Differences in days to heading for nine genotype classes for combinations of *Hd2* and *Ghd7* under NF conditions in the BC_4F_2 population. The genotypes were determined using the closely linked markers RM1357 (*Hd2*) and RM21327 (*Ghd7*). Ko, Hetero, and Ha indicate homozygous for the Koshihikari allele, heterozygous, and homozygous for the Hayamasari allele, respectively. *Numbers* above the *bars* indicate the number of plants belonging to each genotype class. *Bars* represent mean values ± standard deviations. P_{AA}, P_{AD}, P_{DA} and P_{DD} are significant probabilities for AA, AD, DA and DD interactions between *Hd2* and *Ghd7*, respectively

variation in DTH, ranging from 73 to 105 days, and was divided into three groups: 73–80, 84–94, and 96–105 days (Fig. 5b). QTL analysis of heading date demonstrated an association between DTH and genotype markers for the *Hd2* and *Ghd7* regions, but not the markers in other chromosomal regions (Supplemental Table S2). Two-way ANOVA suggested that the effect of the Koshihikari *Hd2* allele was not additive for the different *Ghd7* allele classes ($P < 0.0001$). Orthogonal contrast test showed that all interaction types, AA ($P < 0.01$), AD ($P < 0.01$), DA ($P < 0.01$) and DD ($P < 0.01$) were significant for the interaction of *Hd2* and *Ghd7* under NF conditions. The effect of the Koshihikari *Hd2* allele on heading inhibition differed among the *Ghd7* allele classes. In plants homozygous for the Hayamasari *Ghd7* allele, difference in DTH between plants having homozygous for Koshihikari and Hayamasari allele at *Hd2* was about 13 days, however, in plants homozygous for the Koshihikari *Ghd7* allele, the difference was about only 3 days (Fig. 5c). Similarly, the effect of the Koshihikari *Ghd7* allele on heading inhibition differed among the *Hd2* allele classes. In plants homozygous for the Hayamasari *Hd2* allele, difference in DTH between plants having homozygous for Koshihikari and Hayamasari *Ghd7* was about 23 days, but in plants homozygous for the Koshihikari *Hd2* allele, the difference was about 12 days. These results clearly suggest a genetic interaction in the effect of *Hd2* and *Ghd7*.

Discussion

To clarify the reasons for discrepancies in previously reported detection of *Hd2* between Nipponbare × Kasalath and Koshihikari × Kasalath, we performed a genetic analysis of heading date QTLs using crosses between an extremely early heading cultivar, Hayamasari, and Koshihikari. The results clearly demonstrated that a genetic interaction between *Hd2* and *Hd16* was involved in the previous inconsistent detection of the *Hd2* locus.

Hd2 interacted with *Hd16* under NF and ELD conditions (Fig. 4d, e). Under NF conditions, in plants homozygous for the Koshihikari *Hd16* allele, difference in DTH between *Hd2* Koshihikari allele and Hayamasari allele was small (about 3 days). Therefore, we believe that this interaction between *Hd2* and *Hd16* would decrease the ability to detect *Hd2* under these conditions in Koshihikari genetic background (Ebitani et al. 2005; Yamamoto et al. 2001). A large effect of *Hd2* on heading inhibition (about 13 days)

occurred with the Hayamasari *Hd16* allele. In addition, under both NF and ELD conditions, the effect of *Hd16* depended completely on the Koshihikari *Hd2* allele (Fig. 4d, e), suggesting that *Hd2* and *Hd16* act in the same pathway that controls heading. Although *Hd2* was affected by the *Hd16* allele, a small effect of the *Hd2* allele was observed even with the Koshihikari *Hd16* allele (Fig. 4d, e). These results suggest that the Koshihikari *Hd16* allele was not a null allele or that *Hd2* acts through another pathway to control heading. At present, neither *Hd2* nor *Hd16* have been cloned, so their molecular function must still be analyzed. Elucidation of the molecular functions of these genes will be required to reveal the molecular basis for their interaction. We did not detect *Hd16* in the BILs and the BC_4F_2 population under ELD conditions. However, all five plants homozygous for both the Koshihikari *Hd2* allele and the Hayamasari *Hd16* allele did not head even after 188 days, in contrast with all five plants homozygous for the Koshihikari alleles at both *Hd2* and *Hd16*, which headed after about 133 days (Fig. 4e). These results suggest that *Hd2* accompanied by *Hd16* played a critical role in expressing photoperiod sensitivity. Under ELD conditions, plants homozygous for the Koshihikari allele at *Hd2* and heterozygous at *Hd16* showed large difference in DTH (Fig. 4e). Therefore, it was possible that additional QTL not detected in this study might be involved in a variation in DTH of this BC_4F_2 population. Further analyses of advanced backcross progenies need to be done to make it clear.

Under NF conditions, we also demonstrated an interaction between *Hd2* and *Ghd7* (Fig. 5c), suggesting that both also control heading via the same pathway. The effect of *Hd2* was larger with the homozygous Hayamasari *Ghd7* allele, and the effect of *Ghd7* was larger with the homozygous Hayamasari *Hd2* allele. In previous studies, an interaction between *Hd2* and *Ghd7* was proposed, and our results support that hypothesis. In a BC_1F_1 population of Hoshinoyume × Nipponbare, an epistatic interaction between *qDTH-7-1* and *qDTH-7-2* has been reported for heading (Fujino and Sekiguchi 2005b). In that population, the inhibitory effect of *qDTH-7-2* was observed with the homozygous Hoshinoyume *qDTH-7-1* allele, but not in the *qDTH-7-1* heterozygote, under NF conditions. We hypothesize that *qDTH-7-1* and *qDTH-7-2* correspond to *Ghd7* and *Hd2* based on their chromosomal positions, and the epistatic interaction was similar to that between *Ghd7* and *Hd2* in the present study. Similar interactions have been shown using different cross combinations (Gu and Foley 2007). Based on orthogonal contrast test, all genetic interaction types were significant between *Hd2* and *Ghd7* under NF (Fig. 5c). Further analyses including cloning and expression analysis of *Hd2* will be required to fully explain the genetic interaction between *Hd2* and *Ghd7*. Unfortunately, we could not examine the genetic interaction of *Ghd7* and *Hd2* under ELD conditions. Under ELD conditions, the genetic interaction of *Hd2* and *Hd16* was similar to that of under LD, although additive effect of each QTL was enlarged (Fig. 4d, e). Therefore, we assumed that the genetic interaction of *Hd2* and *Ghd7* is observed not only under LD conditions, but also under ELD conditions. This assumption will also be verified by additional analysis.

Previous study clearly indicated that Hayamasari and Koshihikari have different alleles at *Hd1* (Yano et al. 2000; Nonoue et al. 2008). In fact, under LD and ELD conditions, the chromosomal positions of the QTLs on chromosome 6 were likely to be *Hd1* (Yano et al. 2000), suggesting functional difference in *Hd1* alleles. It should be noted that chromosomal position of the QTL did not match exactly that of *Hd1* (Fig. 3). This may be due to low precision of mapping in QTL analysis. However, we cannot rule out the possibility that additionally another QTL could be detected near *Hd1* that is related to the inhibition of heading under LD and ELD conditions. Fine mapping of the QTL as a single Mendelian factor will be required to clarify their allelic relationship.

In this study, we could not examine the genetic interaction between *Hd2* and *DTH8* (*Hd5*) due to a lack of appropriate advanced progeny. However, a previous study suggested that such an interaction might exist (Gu and Foley 2007). Gu and Foley (2007) showed that *Se7-1*, *Se7-2*, and *Se8*, which appear to be *Ghd7*, *Hd2*, and *DTH8* (*Hd5*), respectively, based on their chromosomal locations, have epistatic effects on rice heading, which suggests that they work in the same pathway. Recently, it was reported that *Ghd8*, which is identical gene to *DTH8* (*Hd5*), was linked in *Ehd1* and *Hd1*-mediated flowering pathway (Yan et al. 2011). All of this information combined suggests that six QTLs (*Hd2*, *DTH8* (*Hd5*) (*Ghd8*), *Ghd7*, *Ehd1*, *Hd1*, and *Hd16*) function in the same genetic control pathway for rice heading. Molecular cloning of several QTLs has revealed several of the genetic control mechanisms in rice heading (*Hd1*, Yano et al. 2000; *Hd6*, Takahashi et al. 2001; *Hd3a*, Kojima et al. 2002; *Ehd1*, Doi et al. 2004; *Ghd7*, Xue et al. 2008; *DTH8*, Wei et al. 2010; *Ghd8*, Yan et al. 2011). Under LD conditions, *Ghd7*, which encodes a CCT (CONSTANS, CONSTANS-LIKE, TIMING OF CAB 1) domain protein, represses the expression of *Ehd1* (Xue et al. 2008). *Ehd1*, a B-type response regulator, promotes the expression of *Hd3a*, a rice florigen gene, and the expression of *RFT1*, an *Hd3a* paralog, whose expression accelerates flowering (Doi et al. 2004; Komiya et al. 2009). The genetic interactions between *Ghd7* and *Hd2* and between *Hd2* and *Hd16* revealed in the present study may therefore suggest the involvement of *Hd2* and *Hd16* in a *Ghd7-Ehd1-Hd3a/RFT1* pathway to repress heading under LD conditions. *Hd1* (Yano et al. 2000) and *Hd2* (Lin et al.

2000) promotes flowering under SD conditions and delays flowering under LD conditions and *Ghd8* showed similar pattern in some genetic background (Yan et al. 2011). This pattern of opposite effect on flowering under SD and LD conditions was similar to that of *Hd16* in the present and previous studies (Matsubara et al. 2008). This may indicate that *Hd16* and *Hd2* function with *Hd1* and/or *DTH8* (*Hd5*, *Ghd8*) in flowering pathway.

The seeds of the Hayamasari × Koshihikari BILs developed in the present study and more information on the genotypes can be obtained from the Rice Genome Resource Center of NIAS (http://www.rgrc.dna.affrc.go.jp/index.html).

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