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ORIGINAL PAPER

Novel QTLs for photoperiodic flowering revealed by using reciprocal backcross inbred lines from crosses between *japonica* rice cultivars

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Abstract The rice *japonica* cultivars Nipponbare and Koshihikari differ in heading date and response of heading to photoperiod (photoperiod sensitivity). Using simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers, we conducted quantitative trait locus (QTL) analyses for heading date in a set of reciprocal backcross inbred lines (BILs) from crosses between Nipponbare and Koshihikari. Under natural-day conditions, transgressive segregation in days to heading (DTH) toward both early and late heading was observed in both BIL populations. QTL analyses revealed that two QTLs—on chromosomes 3 and 6—were involved in the difference in heading date between the parental cultivars. The Nipponbare allele at the QTLs on chromosomes 3 and 6 showed, respectively, increasing and decreasing effects on DTH in both BIL populations. The transgressive segregation observed in the BILs could be accounted for mainly by the complementary action of a set of alleles with opposing effects. Both QTLs were finely mapped as single Mendelian factors in secondary mapping populations (BC_2F_2 plants/BC_2F_3 lines). The QTL on chromosome 3 was mapped in the 1,140-kb interval between 94O03-4 (SSR) and OJ21G19-4 (SNP) and was designated *Hd16*. The QTL on chromosome 6 was mapped in the 328-kb interval between P548D347 (SSR) and 0007O20 (SSR) and was designated *Hd17*. Both *Hd16* and *Hd17* were involved in photoperiod sensitivity, as revealed by observation of the DTH of nearly isogenic lines of Nipponbare under short- and long-day conditions, suggesting that allelic differences in both *Hd16* and *Hd17* account for most of the difference in photoperiod sensitivity between the parental cultivars.

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Introduction

Seasonal changes in daylength, temperature, and rainfall have had a strong influence on the adaptation of many plant species, including crop plants (Stebbins 1950; Harlan 1975). For example, during the process of introduction to higher latitudes, Asian rice cultivars (*Oryza sativa* L.), which are typical short-day plants that originated in tropical areas near the equator, might have undergone changes in heading dates mainly in response to daylength, thereby ensuring maturity during optimal climatic conditions (Vergara and Chang 1985; Oka 1988).

Quantitative trait loci (QTLs) contributing to heading date in *Oryza* species have been intensively explored and mapped to their residing chromosomal regions in many populations derived from crosses between distinct lineages,

such as from *japonica* × *indica* subspecies (Xiao et al. 1996; Yano et al. 1997, 2001; Uga et al. 2007; Gu and Foley 2007; Nonoue et al. 2008; see also Gramene: http://www.gramene.org/), and several QTLs underlying natural variations in heading date have been cloned by a map-based strategy (Yano et al. 2000; Takahashi et al. 2001; Kojima et al. 2002; Doi et al. 2004). *Heading date 1* (*Hd1*) encodes a protein with a zinc finger and the CCT (for CONSTANS, CONSTANS-LIKE, TOC1) motif and is an ortholog of *Arabidopsis CONSTANS* (Yano et al. 2000). *Hd3a* is a rice ortholog of Arabidopsis *FT* and is regulated by *Hd1* (Izawa et al. 2002; Kojima et al. 2002). *Hd6* encodes a casein kinase 2 alpha (Takahashi et al. 2001). A major QTL, *Early heading date 1* (*Ehd1*), encodes a B-type response regulator (Doi et al. 2004). Expression analysis has revealed that *Ehd1* functions upstream of *Hd3a* (Doi et al. 2004), but there may be no ortholog of rice *Ehd1* in Arabidopsis. In addition, recently, Tamaki et al. (2007) clearly demonstrated that Hd3a protein functions as a florigen-type mobile flowering signal. These molecular cloning and expression analyses have revealed that both conserved and diverged mechanisms are involved in the genetic control of flowering (heading) in rice and Arabidopsis; these studies have contributed markedly to our understanding of the genetic control of flowering in rice (Izawa 2007).

A wide range of natural variation in heading date has been observed among *japonica* cultivars. For example, a series of genetic analyses has been conducted on heading date among Japanese cultivars (Okumoto et al. 1991, 1992, 1996; Ichitani et al. 1997, 1998a). These studies identified several loci—*Se1*, *E1*, *E2*, *E3*, and *Ef1*—for heading date. However, the relationships between these previously identified loci and several recently identified QTLs are poorly understood: the low level of genetic diversity among Japanese cultivars means that it is difficult to obtain suitable DNA markers (Kono et al. 2000; Garris et al. 2005; Shirasawa et al. 2007; Zhu et al. 2007, and references therein). Thus, the molecular basis of the natural variation in heading date observed among Japanese cultivars remains unclear.

Among Japanese cultivars, the extremely low frequency of polymorphism has hindered the use of DNA markers to produce efficient genetic analyses, such as QTL analyses. Only one study has reported the use of restriction fragment length polymorphism (RFLP) markers in QTL mapping for cool temperature tolerance at the booting stage (Takeuchi et al. 2001). Recently, plenty of simple sequence repeats (SSR) regions have been identified since the completion of the rice genome sequencing project (IRGSP 2005). This situation has allowed us to effectively screen, on a large-scale, SSR markers showing polymorphism between Japanese cultivars and to perform QTL analyses on their progeny. Thus, a few QTL analyses have been conducted on grain quality (Tabata et al. 2007, Kobayashi et al. 2007) and eating quality (Wada et al. 2006) in a mapping population derived from crosses between Japanese cultivars.

Here, to reveal the genetic basis of differences in heading date between Japanese cultivars, we developed populations of reciprocal backcross inbred lines (BILs) between two cultivars, Nipponbare and Koshihikari. Nipponbare was formerly Japan's leading cultivar, and Koshihikari is the current leading cultivar. Cropping areas of Koshihikari have been extended more northward than those of Nipponbare, suggesting that some traits involving regional adaptability differ between the two cultivars. Possibly, the most important trait is heading date, relating to the maturity of a plant, followed by seed production. Indeed, Nipponbare heads 7–10 days later than Koshihikari in the field under natural day-length (ND) conditions in Tsukuba, Japan. We conducted QTL analyses of heading date in a set of reciprocal BILs and demonstrated that at least two QTLs were involved in the difference in heading date between Nipponbare and Koshihikari under ND conditions. We also revealed that the two QTLs were involved in the photoperiodic response of heading. We report here the chromosomal locations and genetic effects of the QTLs involved in the difference in days to heading (DTH) between Nipponbare and Koshihikari.

Materials and methods

Plant materials and construction of mapping populations

Two Japanese cultivars (*japonica*), Nipponbare and Koshihikari, were used. Two types of mapping population were developed to perform a QTL analysis for heading date in the two cultivars. Nipponbare was crossed with Koshihikari to produce F_1 plants. The resultant F_1 plants were backcrossed with Nipponbare or Koshihikari to obtain reciprocal BC_1F_1 plants. These BC_1F_1 plants were consecutively self-pollinated six times to construct mapping populations or BILs by the single-seed descent method. We constructed 79 lines backcrossed with Nipponbare (N-BILs) and 127 lines backcrossed with Koshihikari (K-BILs).

For a further genetic analysis for putative QTLs, an F_1 population of Nipponbare × Koshihikari was consecutively backcrossed twice with Nipponbare. The resulting BC_2F_1s, which were heterozygous for either target QTL alone, were selected by using DNA markers and self-pollinated to obtain two secondary mapping populations. The BC_2F_2 plants and their self-pollinated progeny were used for linkage mapping of the QTLs detected.

Fine mapping by using advanced backcross progeny

For fine mapping of the detected QTL, we selected one BC_2F_1 plant that was heterozygous for the genomic region containing

the putative QTL but homozygous for other QTLs. Self-pollinated progeny (88 BC_2F_2 plants) from the BC_2F_1 plant were raised. To precisely map the QTL as a single Mendelian factor, we selected BC_2F_2 plants with recombination in the vicinity of the QTL on the basis of the genotypes of the DNA markers used, as well as three F_3 lines as genotype references homozygous or heterozygous for the Nipponbare and Koshihikari alleles. The self-pollinated progeny (F_3 lines) of the plants were used to determine the genotypes at the QTL.

Developing nearly isogenic lines for the detected QTLs

To clarify whether the QTLs detected were involved in photoperiod sensitivity, we developed nearly isogenic lines (NILs) for each QTL detected. An F_1 plant of Nipponbare × Koshihikari and its progeny were consecutively backcrossed four times with Nipponbare, and the resulting BC_4 derivatives were used as NILs. In every backcrossed generation, F_1 plants that heterozygously carried a target QTL region were selected by using DNA markers. In the BC_3 and BC_4 generations, whole-genome surveys were done to select a BC_4F_1 plant in which genomic regions other than the target QTL region were homozygous for Nipponbare alleles. The selected BC_4F_1 plant was subsequently self-pollinated. On the basis of the genotypes of the DNA markers we then selected a BC_4F_2 plant with recombination in the vicinity of the QTL. The self-pollinated progeny BC_4F_3 plants were genotyped by DNA markers to obtain plant homozygous of Koshihikari allele for the target QTL regions. In addition, plant showing homozygous of Nipponbare allele in all DNA markers was also selected and used as isogenic controls.

Scoring of days to heading in mapping populations

The parental cultivars (Nipponbare and Koshihikari), the two BIL populations, and the advanced backcross progeny (BC_2F_2 and BC_2F_3 plants) were grown in a paddy field at the National Institute of Agrobiological Sciences (NIAS) in Tsukuba, Japan, from April to September. The mean daylengths during cultivation period were 13.1 h in 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. The mean temperatures were 11.7°C in April, 17.2°C in May, 20.8°C in June, 23.8°C in July, and 25.7°C in August. We recorded the DTH of each plant as the number of days to the appearance of the first panicle. In the parental cultivars and BILs, DTH was scored in ten plants per line and mean values were calculated for each line. In the advanced backcross progeny, the DTH of each plant was scored.

Evaluation of sensitivity to photoperiod

The parental cultivars and two NILs for the QTL detected were grown under long-day (LD; 14.5 h light; 28°C for 12 h and 24°C for 12 h) and short-day conditions (SD; 10 h light; 28°C for 12 h and 24°C for 12 h) in a controlled-growth cabinet (Especmic TGEH-9, Tokyo, Japan). DTH was scored in ten plants per line and mean values were calculated for each line.

DNA marker analyses

Total DNA was extracted from 2-month-old seedlings of the BC_1F_5 generation by the cetyltrimethylammonium bromide method according to the techniques described by Murray and Thompson (1980).

To obtain SSR markers showing polymorphism between Nipponbare and Koshihikari, we surveyed more than 3,100 genomic regions containing SSR motifs (Chen et al. 1997; Temnykh et al. 2000; McCouch et al. 2002; International Rice Genome Sequencing Project 2005); the resultant informative SSR markers were used for genotyping of both BIL populations. We also used single nucleotide polymorphism (SNP) markers to fill gaps on linkage maps with no SSR marker (Nasu et al. 2002; Monna et al. 2006; see also the PGC SNPs Database System: http://www.pgcdna.co.jp/snps/). Furthermore, in order to map precisely the QTL detected, additional markers—94O03-4 (SSR), OJ21G19-4 (SNP), and 94F01-2 (SSR)—were newly developed in the vicinity of QTL detected on chromosome 3.

For polymerase chain reaction (PCR) of SSR markers, we used a 5-μl reaction volume containing 1 μl template DNA (20 ng μl^{-1}), 0.5 μl 10× PCR buffer, 25 mM $MgCl_2$, 2 mM each dNTP, 0.05 μl *Taq* DNA polymerase (5 U μl^{-1}), 0.1 μl of a 20-pM solution of each primer, and 2.55 μl H_2O. Amplification was performed for 35 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C), followed by 7 min at 72°C. The amplified products were electrophoresed in 3% agarose gel to detect polymorphisms. However, if the difference between the PCR product sizes was too small to discriminate, the PCR products were fluorescently labeled, and their fragment sizes were determined on an ABI 3100 DNA sequencer using Data Collection 2.0 and GeneMapper 3.7 software (Applied Biosystems, Foster City, CA, USA). For SNP markers, we detected SNP using confronting two-pair primers (PCR-CTPP; Hamajima 2001) or the AcycloPrime FP method (Nasu et al. 2002). Information on all the markers used is given in Supplementary Table 1.

Map constructions and QTL analyses of BILs

A set of linkage maps of 111 DNA markers was constructed using N-BILs ($n = 79$) and K-BILs ($n = 127$). Linkage order and genetic distances of marker loci were calculated with MAPMAKER/Exp 3.0 (Lander et al. 1987). Residual heterozygotes were dealt with as missing data (–).

Quantitative trait loci analyses were performed by composite interval mapping (CIM; Zeng 1993, 1994) as implemented by the program Zmapqtl (model 6) of the software package QTL Cartographer version 2.5 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm; see also Basten et al. 2005). CIM was run with a 10-cM window and five background cofactors. Tests were performed at 2-cM intervals, and cofactors were selected via forward–backward stepwise regression with the program SRmapqtl. Genome-wide threshold values ($\alpha = 0.05$) for declaring the presence of QTLs were estimated from 1,000 permutations (Churchill and Doerge 1994; Doerge and Churchill 1996). One-LOD confidence intervals for the positions of the QTLs were defined on the basis of the CIM results.

Results

Difference in photoperiod sensitivity between Nipponbare and Koshihikari

Days to heading of Nipponbare was 116.9 ± 1.6 days; under ND conditions flowering occurred about 7 days later than in Koshihikari (109.8 ± 1.2 days) (Fig. 1a). To examine whether or not the difference in heading date was attributable to a different photoperiod sensitivity, we grew the two cultivars under LD and SD conditions (Fig. 1a, b). Under LD conditions, Nipponbare (94.3 ± 3.3 days) flowered about 24 days later than Koshihikari (70.0 ± 1.2 days), whereas under SD conditions, Nipponbare (47.3 ± 0.7 days) flowered about 4 days earlier than Koshihikari (51.1 ± 1.3 days). The difference in DTH between SD and LD conditions was much larger in Nipponbare (about 47 days) than in Koshihikari (about 19 days). The results suggest that the two cultivars each exhibited a distinct photoperiodic response. It is likely that this difference in photoperiod sensitivity produces the difference in heading date under ND conditions (Fig. 1a, b).

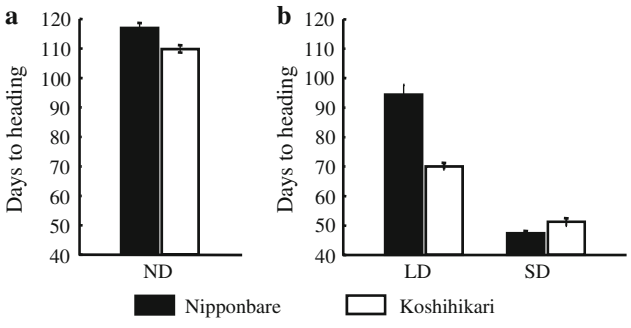

Fig. 1 Days to heading of Nipponbare and Koshihikari under different daylength conditions. **a** Natural daylength (*ND*) conditions, **b** long-day (*LD*) and short-day conditions (*SD*). *Error bars* represent ± 1 standard deviation

Variation in DTH in the two BILs

We constructed a set of mapping populations consisting of 79 N-BILs and 127 K-BILs. Under ND conditions, DTH ranged from 102.1 to 123.0 days in N-BILs and from 102.7 to 125.0 days in K-BILs (Fig. 2). In both populations, continuous variations and transgressive segregations were observed in the directions of both earlier and later than the parental cultivars Koshihikari (109.8 days) and Nipponbare (116.9 days).

Construction of linkage maps and detection of QTLs for DTH

Of the publicly available SSR markers extensively screened, about 11.8% showed polymorphism between Nipponbare and Koshihikari. The distribution of these informative SSR markers on the 12 rice chromosomes was not even, so we selected 102 of these informative SSR markers to develop a linkage map (Supplementary Figure 1 and Supplementary Table 1). In addition, nine SNP markers showing polymorphism between Nipponbare and Koshihikari were chosen (PGC SNPs Database System: http://www.pgcdna.co.jp/snps/) (Supplementary Figure 1 and

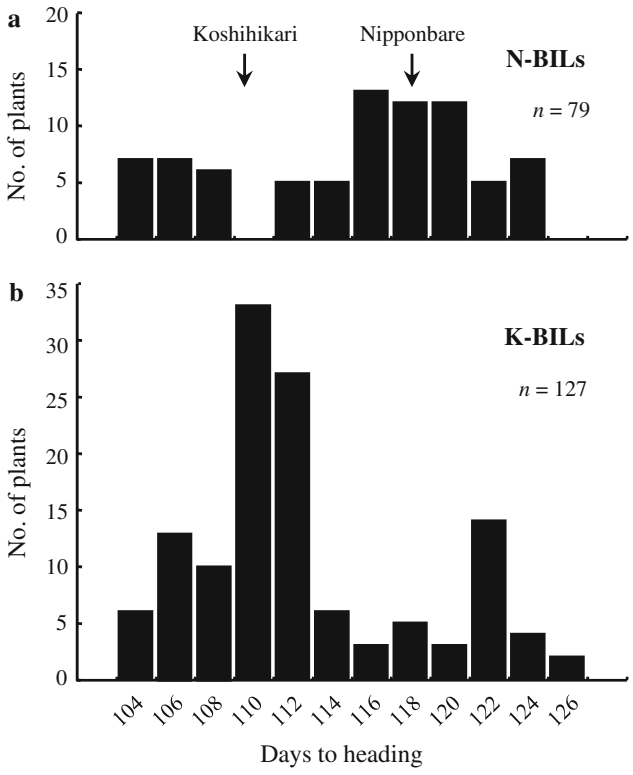

Fig. 2 Distributions of days to heading (DTH) under natural daylength conditions in N-BILs (**a**) and K-BILs (**b**). The average values of DTH were 116.9 ± 1.6 days (Nipponbare) and 109.8 ± 1.2 days (Koshihikari), as marked by *arrows*

Supplementary Table 1). Linkage maps were constructed by using in total 111 SSR and SNP markers. The total map length was 973.1 cM in N-BILs and 956.1 cM in K-BILs. The genome coverage of linkage maps constructed in this study was 93.6% (357.9 Mb/382.2 Mb), as estimated by the physical positions of the markers at the distal end of each chromosome (Supplementary Table 1). The average interval between adjacent markers was 8.8 cM in N-BILs and 8.6 cM in K-BILs—a physical distance of 3.2 Mb (Supplementary Table 1). The average amount of missing data across the 111 marker loci was 3.7% in N-BILs and 6.1% in K-BILs. Although there were several chromosomal regions where DNA markers were sparsely mapped, in general these linkage maps covered all of the rice genome. These maps were therefore suitable for QTL detection.

Quantitative trait loci analyses for DTH were performed in the two BIL populations under ND conditions. Two putative QTLs were detected, on chromosomes 3 and 6, in both populations (Fig. 3 and Table 1). One QTL was mapped near 87C10-17 on chromosome 3 in both BILs. The phenotypic effect of this QTL was relatively large; additive effects for N-BILs and K-BILs were 5.4 and 5.8 days and the R^2 values were 0.63 and 0.67, respectively. The Nipponbare allele showed an increasing effect on DTH. The other QTLs were mapped near 0007O20 on chromosome 6 in both BILs and had a relatively small effect on DTH. In these QTLs, additive effects were −2.6 and −2.7 days and the R^2 values were 0.13 and 0.17, respectively, for N-BILs and K-BILs and the Nipponbare allele showed a decreasing effect on DTH. In addition, although there was not strong statistical support, we could not completely rule out the existence of a QTL with minor effect (LOD 2.5 and 5.7; additive effect 1.1 and 1.0 days; R^2 0.02 and 0.03; in N-BILs and K-BILs, respectively) near SNP marker S0146-05 on chromosome 8 (Supplementary Figure 2).

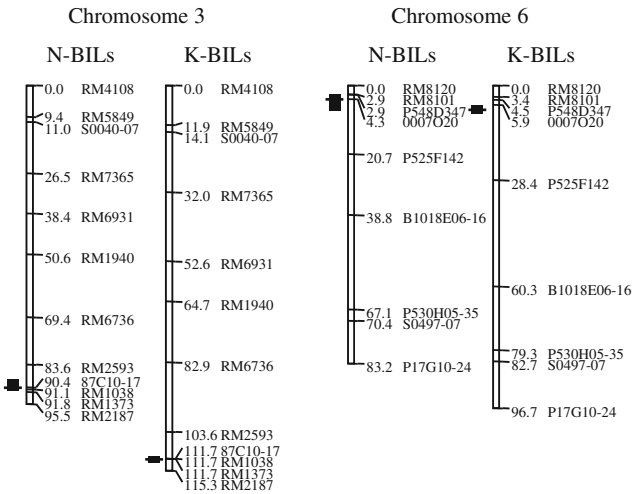

Fig. 3 Quantitative trait loci detected by using a set of reciprocal BILs. *Vertical bars* to the *left* of each linkage map of chromosomes indicate one-LOD confidence interval, and *horizontal bars* indicate the position of the LOD peak at each QTL. Distances are shown in centimorgans

To test whether digenic interaction was involved in expression of the detected QTLs, we compared DTH under ND conditions among four genotype classes of the nearest marker locus to both QTLs. Two-way ANOVA revealed the genetic effect of both QTLs in all genotype classes, although the interaction was significant in only one of the BIL populations ($P < 0.0034$ in K-BILs). These results suggest that allele-specific digenic interaction of the two QTLs was not greatly involved in the phenotypic variation of heading date in the BILs.

Verification and fine mapping of the QTL on the long arm of chromosome 3

For fine mapping of the QTL on the long arm of chromosome 3, we selected one BC_2F_1 plant that was heterozygous on the long arm of chromosome 3 containing the putative QTL, but homozygous on the short arm of chromosome 6 containing the other QTL. The self-pollinated progeny—88 BC_2F_2 plants—showed a bimodal DTH distribution from 102 to 116 days under ND conditions (Fig. 4a). A QTL linked with the SNP marker OJ21G19-4 accounted for a large part of the total phenotypic variation ($R^2 = 0.89$) in this BC_2F_2 population. The additive effect of the Nipponbare allele at the QTL was 5.4 days. On the basis of the genotype of OJ21G19-4, we classified the F_2 plants as homozygous for the Nipponbare or Koshihikari allele or heterozygous (Fig. 4a). F_2 plants homozygous for the Koshihikari allele at OJ21G19-4 headed earlier

Table 1 QTLs detected for days-to-heading in the two BILs

Population	Chr.	Marker interval	Nearest marker	LOD score	*a*	R^2
N-BILs	3	RM2593–87C10–17	87C10–17	30.3	5.4	0.63
	6	P548D347–P525F142	0007O20	11.7	−2.6	0.13
K-BILs	3	RM2593–RM2187	87C10–17	63.0	5.8	0.67
	6	P548D347–P525F142	0007O20	33.5	−2.7	0.17

The significance thresholds for an experiment-wide error rate of $\alpha = 0.05$ were 5.4 LOD in the N-BILs and 8.1 LOD in the K-BILs, respectively

a is the additive effect, the sign of the additive effect corresponds to the direction of the effect of the Nipponbare allele. R^2 is the proportion of variance explained by the QTL

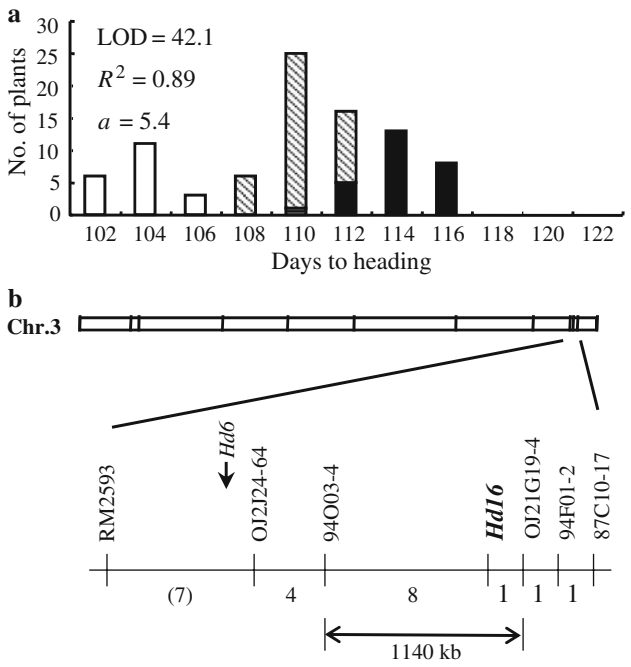

Fig. 4 Fine linkage mapping of *Hd16* in advanced backcross progeny. **a** Distribution of days to heading (DTH) in a BC_2F_2 population. LOD (log_{10} likelihood ratio), R^2 (proportion of variance explained), and *a* (additive effect of Nipponbare allele) for the nearest marker of DTH were determined by QTL Cartographer. *Black color* indicates homozygous for Nipponbare allele, *white color* indicates homozygous for Koshihikari allele, *shaded region* indicates heterozygous. **b** Chromosomal location of *Hd16*. *Markers* used are indicated above the linkage map, and the number of recombinants is shown below. The number of recombinants between RM2593 and OJ2J24–64 was obtained from the BC_2F_2 population. *Hd6* is located in the interval between the markers RM2593 and OJ2J24–64, according to the *Hd6* sequence (adapted from Takahashi et al. 2001)

(≤106 days) than did those homozygous for the Nipponbare allele (≥110 days). DTH of the heterozygous plants was intermediate between those of the homozygotes, but the distribution was shifted toward late heading (Fig. 4a). These results clearly confirm the existence of the QTL on the long arm of chromosome 3; the Nipponbare allele at the QTL increased DTH in a semi-dominant manner.

To precisely map the QTL as a single Mendelian factor, on the basis of the genotypes of the SSR and SNP markers used we selected 14 BC_2F_2 plants with recombination in the vicinity of the QTL, as well as three F_3 lines as genotype references (Supplementary Table 2). The self-pollinated progeny (F_3 lines) of the plants were used to determine the genotypes at the QTL. The F_3 lines could clearly be classified into three DTH classes: three lines showed early heading; four lines showed late heading; and seven lines showed wide variation in DTH (Supplementary Table 2). These three patterns corresponded perfectly to the genotypes of the QTL homozygous for the Koshihikari allele, homozygous for the Nipponbare allele, and heterozygous, respectively. Thus, the QTL was mapped between SSR marker 94O03-4 (physical position 32,855 kb on chromosome 3 of rice genome build 4; http://rgp.dna.affrc.go.jp/J/IRGSP/Build4/build4.html) and SNP marker OJ21G19-4 (physical position 33,996 kb) on chromosome 3 (Fig. 4b). This interval corresponded to 1,140 kb. According to these results, we designated this QTL *Heading date 16* (*Hd16*).

Verification and fine mapping of the QTL on the short arm of chromosome 6

For fine mapping of the QTL on the short arm of chromosome 6, we selected one BC_2F_1 plant that was heterozygous on the short arm of chromosome 6 containing the putative QTL, but homozygous on the long arm of chromosome 3 containing the other QTL. The self-pollinated progeny—88 BC_2F_2 plants—showed a unimodal DTH distribution from 114 to 122 days under ND conditions (Fig. 5a). A QTL linked with the SSR marker 0007O20 explained a large part of the total phenotypic variation ($R^2 = 0.60$) in this BC_2F_2 population. The additive effect of the Nipponbare allele at the QTL was −2.0 days. On the basis of the genotype of 0007O20, we classified the F_2 plants into three genotype classes (Fig. 5a). F_2 plants homozygous for the Nipponbare allele at 0007O20 headed earlier (≤118 days) than did those homozygous for the Koshihikari allele (≥118 days). DTH of the heterozygous plants was distributed over the ranges of the homozygotes (Fig. 5a). These results clearly confirm the existence of the QTL on the short arm of chromosome 6, and the Koshihikari allele at the QTL increased DTH in a semi-dominant manner.

For precise mapping of the QTL as a single Mendelian factor, on the basis of the genotypes of the SSR markers used we selected 25 BC_2F_2 plants with recombination in the vicinity of the QTL, as well as three F_3 lines as genotype references (Supplementary Table 3). The self-pollinated progeny (F_3 lines) of the plants were used to determine the genotypes at the QTL. The F_3 lines could clearly be classified into three DTH classes: 11 lines showed early heading, six lines showed late heading, and eight lines showed wide variation in DTH (Supplementary Table 3). These three patterns agreed well with the genotypes of the QTL homozygous for the Nipponbare allele, homozygous for the Koshihikari allele, and heterozygous, respectively. Thus, the QTL was mapped between two SSR markers, P548D347 (1,964 kb on chromosome 6) and 0007O20 (2,292 kb), on chromosome 6 (Fig. 5b). This interval was 328 kb long. On the basis of the above results, we designated this QTL *Heading date 17* (*Hd17*).

Photoperiodic response of *Hd16* and *Hd17*

Comparison of DTH of the parental cultivars grown under LD and SD conditions revealed that Nipponbare responded

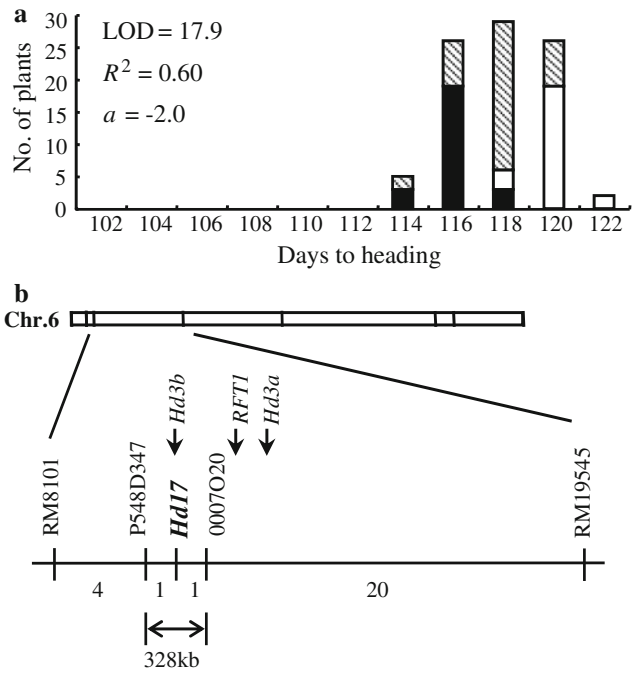

Fig. 5 Fine linkage mapping of *Hd17* in advanced backcross progeny. **a** Distributions of days to heading (DTH) in a BC_2F_2 population. LOD (log_{10} likelihood ratio), R^2 (proportion of variance explained), and *a* (additive effect of Nipponbare allele) for the nearest marker of DTH were determined by QTL Cartographer. *Black color* indicates homozygous for Nipponbare allele, *white color* indicates homozygous for Koshihikari allele, *shaded region* indicates heterozygous. **b** Chromosomal location of *Hd17*. *Markers* used are indicated above the linkage map, and the number of recombinants is shown below. *Hd3a* and *RFT1* are located in the interval between markers 0007O20 and RM19545 according to their sequences (adapted from Kojima et al. 2002), and *Hd3b* is located in the interval between RM8101 and 0007O20 according to RFLP marker R1952 previously used (adapted from Monna et al. 2002)

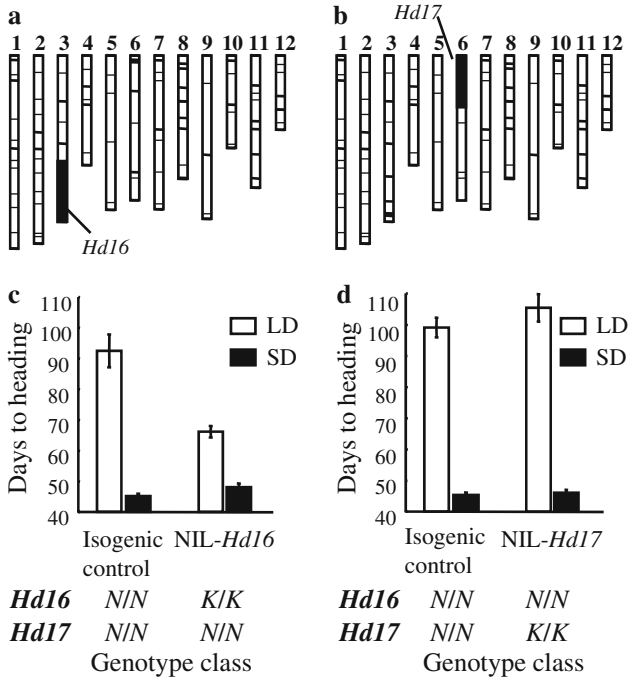

Fig. 6 Days to heading (DTH) of NILs for *Hd16* and *Hd17* in a genetic background of Nipponbare. Graphical representations of genotype of NIL-*Hd16* (**a**) and NIL-*Hd17* (**b**). The 12 *vertical boxes* are the rice chromosomes, *horizontal bars* indicate the positions of SSR markers used for genotyping of the target region and background. Differences in DTH between NIL-*Hd16* and the isogenic control (**c**) and between NIL-*Hd17* and the isogenic control (**d**) under LD and SD conditions. *N* and *K* are the Nipponbare and Koshihikari alleles, respectively. *Error bars* represent ±1 standard deviation

more strongly to photoperiod than did Koshihikari (Fig. 1b). The result raised the question of whether both *Hd16* and *Hd17*, or either alone, were involved in the photoperiodic response of heading. To answer this question, we developed NILs for *Hd16* and *Hd17* by marker-assisted selection (MAS) (Fig. 6a, b). Although, in the NIL selected, large regions on chromosomes 3 and 6 were substituted in the genetic background of Nipponbare, no other region was likely to have been substituted. Thus, these lines could be used as NILs in the analysis.

We raised these two NILs as well as Nipponbare-type plants (isogenic controls) and compared the DTH values under LD and SD conditions (Fig. 6c, d). Under LD conditions, NIL-*Hd16* (66.1 ± 1.9 days) flowered about 26 days earlier than the isogenic control (92.4 ± 5.3 days), whereas under SD conditions NIL-*Hd16* (48.4 ± 1.3 days) flowered about 3 days later than the isogenic control (45.1 ± 0.8 days). The difference in DTH between SD and LD conditions was much smaller in NIL-*Hd16* (about 18 days) than in the isogenic control (about 47 days). The result suggests that *Hd16* is likely to be involved in photoperiod sensitivity. This pattern of difference in DTH in response to distinct differences in daylength resembles that between Nipponbare and Koshihikari (Fig. 1).

In contrast, NIL-*Hd17* (105.3 ± 4.4 days) flowered about 6 days later than the isogenic control (99.0 ± 3.2 days) under LD conditions, but there was no significant difference in DTH between NIL-*Hd17* (46.0 ± 0.9 days) and the isogenic control (45.3 ± 0.8 days) under SD conditions. From these observations it was difficult to conclude that *Hd17* is involved in photoperiod sensitivity.

Discussion

In general, it has not been easy to perform genetic analysis using DNA markers among *japonica* cultivars—in particular Japanese cultivars—owing to their extremely low frequency of polymorphism. However, recent progress in entire rice genome sequencing has allowed us to explore SSR and SNP markers exhibiting polymorphism among closely related cultivars. Here, we used polymorphic SSR

and SNP markers to successfully construct linkage maps in a set of reciprocal BILs from crosses between the Japanese cultivars Nipponbare and Koshihikari. QTL analyses of heading date using two BILs revealed that at least two QTLs, *Hd16* and *Hd17*, were involved in variation in heading date of BILs. Although involvement of an additional QTL could not be ruled out (in fact, it is possible that a minor QTL exists near the SNP marker S0146-05 on chromosome 8), most of phenotypic variations in both BILs could be explained by these two QTLs. As QTL detection is often affected by epistatic interaction with other background factors (Yamamoto et al. 2000; Lin et al. 2000; Yano 2001), we developed two reciprocal BILs between Nipponbare and Koshihikari to detect such epistasis. However, we detected two QTLs in both populations, and the estimated genetic parameters—additive effects and R^2—had similar values for each corresponding QTL, suggesting that expression of these two QTLs is not affected by interaction with factors in the parental genetic background. The LOD value for both QTLs was higher in K-BIL than in N-BIL (Table 1). This difference might be due to the difference in population size.

Numerous data on heading date QTLs, such as their chromosomal locations and genetic effects, have been published in the last decade and summarized in the public database Gramene (http://www.gramene.org/). It would be interesting to compare the chromosomal locations of these QTLs and those examined here. However, it has often proved difficult to compare precisely the chromosomal locations of these previously identified QTLs. Because many QTLs had been identified only by QTL mapping, their precision and reliability vary with the numbers of markers and plants used in the mapping populations. However, some QTLs for heading date have been mapped with high accuracy or molecularly cloned, making comparison possible. The chromosomal location of *Hd16*, mapped in the 1140-kb interval between 94O03-4 and OJ21G19-4, was obviously different from that of *Hd6*, which encodes the α subunit of protein kinase CK2, because *Hd6* is located 495 kb centromere side of 94O03-4 (Fig. 4b; Takahashi et al. 2001). Moreover, sequencing analysis revealed that the Koshihikari allele of *Hd6* had a SNP generating a premature stop codon as Nipponbare (Takahashi et al. 2001), demonstrating that Koshihikari *Hd6* was non-functional as that of Nipponbare. These results clearly demonstrated that *Hd16* is a different locus from *Hd6*. In contrast, there are several QTLs, which have been finely mapped as single Mendelian factors, in the region of *Hd17* on the distal end of the short arm of chromosome 6. From its map location, *Hd17* (the 328-kb interval between P548D347 and 0007O20) was clearly different from the previously cloned heading date genes *RFT1* and *Hd3a,* which are orthologs of *Arabidopsis FT*, because *RFT1* and *Hd3a* are located 634 and 647 kb upstream of 0007O20 (Fig. 5b; Kojima et al. 2002; Izawa et al. 2002). However, *Hd17* was mapped at the same chromosomal region as *Hd3b*, which was finely mapped and co-segregated with RFLP marker R1952 in the advanced backcross progeny of Nipponbare and Kasalath (*indica* cultivars) (Monna et al. 2002). The Kasalath allele at *Hd3b* has the effect of increasing DTH against the Nipponbare allele, in the same way as the Koshihikari allele did, under LD and ND conditions. Therefore, it is likely that *Hd17* corresponds to *Hd3b*. To verify this allelic relationship between *Hd17* and *Hd3b* it will be necessary to clone one of these and compare the sequences of both genes.

A series of genetic analyses have been conducted without DNA markers on the variation in heading date among Japanese rice cultivars, and several loci—*E1*, *E2*, *E3*, *Se1*, and *Ef1*—have been found (Okumoto et al. 1991, 1992, 1996; Ichitani et al. 1997, 1998a). Some of the allelic relationships between those loci and QTLs were clarified. For example, *Hd1* is identical to *Se1* on chromosome 6 (Yano et al. 2000). *Ehd1* occupies the same locus as *Ef1* on chromosome 10 (Doi et al. 1998, 2004). Sequence comparison has revealed that *Hd6* on chromosome 3 is likely to be *E3* (Y. Takahashi and M. Yano, unpublished data). Furthermore, *E1* might be allelic with *Hd4* on chromosome 7 (Ichitani et al. 1998b; Lin et al. 2003). In contrast, Okumoto et al. (1992) have proposed that the difference in heading date between Nipponbare and Koshihikari may be due to an allelic difference at the *E2* locus: Koshihikari carries an allele dominant to that of Nipponbare. Therefore, *E2* might correspond to either *Hd16* or *Hd17*. Unfortunately, the chromosomal location of *E2* is still unknown. Further analyses, such as cloning and sequence comparison, will be required to ascertain the relationship between these loci.

Our QTL analyses revealed that the difference in DTH between Nipponbare and Koshihikari under ND conditions was explained mainly by the two QTLs, *Hd16* on chromosome 3 and *Hd17* on chromosome 6. Progeny (e.g., F_2, RILs, or BILs) derived from a given cross combination often showed extreme phenotypic values exceeding that of either parental line. This phenomenon is referred to as transgressive segregation (Grant 1975). In general, transgressive segregation is generated by a set of alleles with opposing effects from both parental lines. We observed transgressive segregation here, and it could have been caused by the combination of alleles at *Hd16* and *Hd17*. Such genetic architecture may well drive the discovery of new ecological niches or cropping areas made possible through hybridization, even if the phenotypic difference between parents is small (Rieseberg et al. 2003). We were able to map *Hd16* and *Hd17* as single Mendelian factors by using advanced backcross progeny, and demonstrated that a wide range of variation in heading date could be generated

by a combination of alleles of these two loci. It is noteworthy that the allelic difference in *Hd17* between Nipponbare and Koshihikari was less than 3 days; yet even this small difference can be manipulated as part of a fine-tuning process by MAS in rice breeding programs.

In several QTL mappings and cloning of QTLs for heading date, digenic epistatic interactions have generated a wide range of variation in heading date (Lin et al. 2000; Yamamoto et al. 2000). From the ANOVA results we could not conclusively rule out the existence of epistasis between *Hd16* and *Hd17*. The failure to rule out epistasis might be due to allelic difference in *Hd16* and *Hd17* between Nipponbare and Koshihikari. The results obtained in the QTL analysis do not explain the level of function of alleles at either locus. Assuming that the phenotypic difference between the two sets of alleles is relatively small, the alleles in both *Hd16* and *Hd17* might not show loss of function. The allelic differences in the two genes between the parents might be caused instead by modification of the alleles to produce, for example, a single amino acid change (generating a slightly less functional allele) (Yano 2001; Alonso-Blanco et al. 2005; Doebley et al. 2006). Accordingly, the phenotypic variation produced by epistasis between *Hd16* and *Hd17*, if any, would be obscure and thus difficult to detect clearly. Therefore, the cloning and identification of functional differences in the genes is a prerequisite for examining whether epistasis between the two genes contributes to the variation in DTH. Moreover, it has been verified that *Hd1* is involved in the photoperiodic response, which is characterized as an increase in DTH under LD conditions and a decrease in DTH under SD conditions in plants that harbor *Hd1* from Nipponbare (Yano et al. 2000). Sequence of Koshihikari *Hd1* (Acc. No. AB375859) is identical with that of Nipponbare (Accession No. AB041837) (Yano et al. 2000), indicating that Koshihikari carries a functional allele at the *Hd1* locus. In this study, it was clearly demonstrated that response to daylength was disrupted in NIL-*Hd16* (Fig. 6). Based on these results, it was speculated that a digenic interaction between *Hd1* and *Hd16* might be involved in expression of photoperiod sensitivity. This should be proved by developing a series of NIL and observing phenotypic effect of *Hd16* in the background of loss of function of *Hd1*.

Our results suggest that the two QTLs, *Hd16* and *Hd17*, are involved in photoperiodic response of heading in rice. Several genes for photoperiodic response have been identified from QTL analyses and map-based cloning (Izawa et al. 2003; Izawa 2007). From genetic analyses of Nipponbare and Kasalath, several NILs have been developed and used to clarify that *Hd1*, *Hd2*, *Hd3a*, *Hd5*, and *Hd6* are involved in photoperiodic response of heading. Combination of two particular alleles of these loci in one background has allowed us to verify the epistatic interaction between these loci (Lin et al. 2000, 2003; Yamamoto et al. 2000; Nonoue et al. 2008). We also developed two NILs for *Hd16* and *Hd17*. To deepen our understanding of the genetic control of photoperiodic response in heading in rice, it would be interesting to prove the involvement of epistatic interaction by developing combined and pyramiding lines of these loci.

The genetic and molecular bases of heading date have been greatly clarified during the last decade (reviewed by Yano et al. 2001; Izawa et al. 2003; Hayama and Coupland 2004; Izawa 2007). Several studies contributed to our understanding of the genetic control of heading in rice, and four QTLs were cloned at the molecular level (*Hd1*, Yano et al. 2000; *Hd6*; Takahashi et al. 2001; *Hd3a*, Kojima et al. 2002; *Ehd1*, Doi et al. 2004), and to our understanding of heading date in rice (Yano et al. 2001). Furthermore, they have had an impact on the comparative biology of plant flowering and on rice breeding (Izawa et al. 2003). However, several QTLs for heading date remain to be identified, although here we detected two QTLs. Of these two, *Hd16* is definitely different from *Hd6*, which has been cloned. We mapped *Hd16* as a single Mendelian factor by using advanced backcross progeny, demonstrating that map-based cloning of *Hd16* will be feasible. Furthermore, although the allelic relationship between *Hd3b* and *Hd17* is still unclear, our detection of *Hd17* opens up the possibility of map-based cloning of additional flowering-time genes. Although we were able to delimit the candidate genomic regions of *Hd16* and *Hd17* to within 1,140 and 328 kb, respectively (Figs. 4b, 5b), there are, respectively, 150 and 49 predicted genes in the candidate regions of the QTLs, as determined from an examination of the rice genome annotation database (Ohyanagi et al. 2006, Rice Annotation Project 2008). It would be difficult to identify a candidate gene from the annotation, and further efforts will be required to identify these QTLs at the molecular level. In general, it has been necessary to process large numbers of plants (1,000 to 3,000) for the map-based cloning of QTLs. Because of the difficulty in identifying candidate genes from large genomic regions, delimitation of the candidate genomic region to as close an extent as possible is the most important aspect of map-based cloning. Large-scale sequencing technologies have recently been established (Blow 2007; Hutchison 2007). Because of these advances, it is now feasible to sequence large candidate genomic regions—even greater than 1 Mb. In the case of *japonica* × *japonica* crosses, such as Nipponbare × Koshihikari, this feature is often a disadvantage for linkage mapping, but once we delimit the candidate genomic region of the target gene, a low level of polymorphism turns out to be advantageous. We expect definition of the causal sequence polymorphism within the candidate region to be easier between *japonica* cultivars than between genetically distinct lineages, such as

between *japonica* and *indica* cultivars, which are highly polymorphic from one another. Elucidation of the structure and function of genes of interest will lead us to a better understanding of the molecular nature of photoperiodic control in rice.

In general, as heading date is a very important factor for the adaptability of cultivars, the modification of heading date in a particular cultivar has several implications for rice breeding. As elevation of temperature at the ripening stage in rice has caused serious problems in Japan by decreasing grain quality and yield (Morita 2000; Kobayashi et al. 2007; Tabata et al. 2007), shifting the heading date is one solution for avoiding these high temperatures. MAS has been used in the introgression of desirable alleles at QTLs and in the development of several isogenic lines as new rice cultivars (Takeuchi et al. 2006; Neeraja et al. 2007). Here, we detected new QTLs, *Hd16* and *Hd17*, for heading date and identified two different alleles at each QTL. The allelic difference in DTH at *Hd16* was relatively large (about 10 days). Although the heading date of Koshihikari has already been shifted through MAS by using some alleles from a distantly related *indica* cultivar, Kasalath (Takeuchi et al. 2006), here we successfully provided a new gene source for use in MAS to develop early- or late-heading cultivars.

Seeds and genotype data of the reciprocal BILs used here are available at the Rice Genome Resource Center at NIAS (http://www.rgrc.dna.affrc.go.jp/index.html.en).

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