Research Paper

Detection of novel QTLs *qDTH4.5* and *qDTH6.3*, which confer late heading under short-day conditions, by SSR marker-based and QTL-seq analysis

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Heading date is one of the most important traits in rice breeding. It is governed by multiple genes, including known quantitative trait loci (QTLs). In general, almost all *japonica* cultivars, including Nipponbare, head early under short-day (SD) conditions, but some *indica* cultivars, including Kasalath, head late. To explain this difference, we identified QTLs controlling heading date under SD conditions. We used NILs, CSSLs, and BILs from a cross between Nipponbare and Kasalath, and evaluated days to heading (DTH) under SD conditions. No NILs or CSSLs showed late heading, but two BILs (BIL-55 and BIL-78) had almost the same DTH as Kasalath. We developed an F₂ population from a cross between BIL-55 and Nipponbare and performed QTL analysis using SSR markers. The late-heading phenotype was controlled by two known genes and at least two novel QTLs on chromosomes 4 and 6, named *qDTH4.5* and *qDTH6.3*. These QTLs were confirmed by QTL-seq. The QTLs and polymorphisms detected here will provide useful information for further genetic studies and breeding under SD conditions at lower latitudes.

Key Words: heading date, rice, short-day conditions, QTL, QTL-seq, whole-genome re-sequencing.

Introduction

Rice is the most important staple food for around half of the world's population. The FAO forecasts that the global population will increase from 7.3 billion people in 2015 to 9.6 billion by 2050, and peak at about 10.9 billion by 2100 (FAOSTAT). A recent analysis demonstrates that global food demand in 2050 will be double that of 2005 (Tilman *et al.* 2011). Since rice is a major food in tropical and subtropical Asia, where the population will increase greatly, the enhancement of rice production is one of the most crucial issues for the near future.

Despite research on how to increase rice yields, a lack of adaptation by farmers to climate change and limited dissemination of accurate knowledge will cause global crop yields

in 2050 to be 6.9% below estimated yields in the absence of climate change (Wiebe *et al.* 2015). The coming decades are expected to see further increases in temperature, raising the risks of more intense damage by pests and disease, water shortages, and extreme weather events. In addition to climate change, available agricultural land is also decreasing owing to such as rapid urbanization and heavy metal pollution derived from industrial activities. Therefore, it is necessary not only to breed new cultivars with tolerance to biotic and abiotic stresses, but also to develop cultivars adapted to a variety of agricultural systems. However, even recent advances in understanding physiological regulation of stress tolerance and rice yield at the molecular level will not be enough to produce and enhance a sustainable food supply. One solution to the problem is multiple cropping to use the limited arable land more efficiently.

Rice is a facultative short-day (SD) plant, therefore it flowers (= heads) and matures early under SD conditions (Suenaga 1936). Most traditional cultivars in tropical and subtropical Asia mature in 160–170 days owing to strong

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photoperiod sensitivity. These are suitable for single cropping but not for multiple cropping (Khush 2001). Even IR8 and later cultivars, which mature in 130 days (Khush 1995), do not leave sufficient time in the rainy season to grow another crop of rice. Therefore, improved cultivars with rapid vigor and even shorter growth duration were developed by incorporating early-heading genes for early maturity (Khush 2001). In general, almost all *japonica* cultivars, including Nipponbare, head early under SD conditions, but some *indica* cultivars, including Kasalath, head late (Vergara and Chang 1985). Thus, *japonica* cultivars are essential as a genetic resource to improve heading date under SD conditions. However, most previous studies of heading date were performed in temperate regions or at high latitudes, where the daylength during the summer season is long-day (LD) condition, exceeding the critical daylength for *japonica* cultivars, and thus little is known about the control of heading date at the molecular level under SD conditions.

Recent remarkable advances in molecular genetics and biology have made it possible to identify and characterize quantitative trait loci (QTLs) or genes for heading date. QTL and mutant analysis, respectively, have been used to detect genes underlying genetically complex traits, including days to heading (DTH) in rice (Lee and An 2015).

Recent developments in whole-genome sequencing have accelerated the analysis of QTLs. Takagi *et al.* (2013) identified plant QTLs by whole-genome re-sequencing of two DNA bulks of progeny showing extreme phenotypic values, using next-generation sequencing technology. This method, named QTL-seq, does not require DNA marker development and genotyping, and thus allows rapid identification of QTLs. Further, since QTL-seq is based on whole-genome re-sequencing, it can determine not only the genotype of the undefined genetic region by SSR (simple sequence repeat) or the Illumina GoldenGate assay, but also all polymorphisms within genes in candidate QTL regions. For example, Lu *et al.* (2014) performed QTL-seq for flowering time QTLs in cucumber, and identified a homolog of the *Arabidopsis* *FLOWERING LOCUS T (FT)*.

In this study, we evaluated heading date under SD conditions, using backcross inbred lines (BILs), chromosome segment substitution lines (CSSLs), and near-isogenic lines (NILs), derived from crosses between the early-heading cultivar Nipponbare and the late-heading cultivar Kasalath. We found two BILs with almost the same DTH as Kasalath, and focused on one of them, BIL-55. QTL analysis using an F₂ population derived from a cross between Nipponbare and BIL-55 identified four QTLs. Two of these corresponded to *Hd1* and *Hd3a*. We have named the other two *qDTH4.5* and *qDTH6.3*. Our results will be helpful in elucidating heading date regulation under SD conditions and in breeding short-growth-duration cultivars for use in multiple cropping systems.

Materials and Methods

Plant materials

We used a set of 98 BILs (Lin *et al.* 1998) and 54 CSSLs (<http://www.rgrc.dna.affrc.go.jp/ineNKCSSL54.html>) developed from a cross between *japonica* Nipponbare and *indica* Kasalath, backcrossed to Nipponbare, provided by the Rice Genome Resource Center (www.rgrc.dna.affrc.go.jp/stock.html). The BILs and CSSLs were selected by the respective use of 245 and 214 restriction-fragment-length polymorphism markers. To confirm the effects of putative QTLs for heading date under SD (10L/14D) conditions, we investigated the heading dates of five plants per line in a growth chamber under the temperature of 28°C during light periods and 24°C during dark periods, and a metal halide lamp at a photosynthetic photon flux density of 450 to 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. To confirm the effect of known rice heading date genes or QTLs, we then grew 23 NILs in the growth chamber as before. Each NIL carried the heading date gene(s) *Hd1*, *Hd2*, *Hd3a*, *Hd4*, *Hd5*, *Hd6*, *Hd7*, *Hd8*, *Hd9*, *Hd10*, *Hd11*, *Hd12*, *Hd13*, *Hd14* and *Hd15* introgressed from Kasalath (Doi *et al.* 2004, Kojima *et al.* 2002, Lin *et al.* 1998, 2002, 2003, Takahashi *et al.* 2001, Uga *et al.* 2003, Yano *et al.* 2000).

Evaluation of heading date

The day when the first panicle of a plant became exposed was scored as the heading date of a line. QTL analysis used the value of DTH from sowing. We measured DTH of five plants per line under SD conditions and used the mean.

QTL analysis using BIL population

The linkage map was based on data held at the Rice Genome Resource Center (BILs: www.rgrc.dna.affrc.go.jp/jp/ineKKBIL182.html; CSSLs: www.rgrc.dna.affrc.go.jp/jp/ineKCCSSL39.html). Information on 242 markers in 98 BILs was available. To detect QTLs, we used composite interval mapping (CIM). To map QTLs, we used R/qtl software (<http://www.rqtl.org/>). QTLs for BILs were considered significant at $P < 0.05$, with an LOD score calculated from 1000 permutations.

Genotyping by GoldenGate assay

Genomic DNA was extracted from 5 late BIL lines including BIL-55, Nipponbare, and Kasalath by the CTAB method (Stewart and Via 1993). The DNA was used for genotyping by the GoldenGate Bead Array Technology Platform and the BeadStation 500G system (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. A subset of 384 SNP markers was used to cover the entire genome according to their map location (Ebana *et al.* 2010). The genomic DNA of the parents was screened with these markers; 289 markers were polymorphic between the parents. Those showing polymorphism between Nipponbare and late BILs are shown in **Supplemental Table 1**.

Screening of SSR markers

From previously reported rice SSR markers (McCouch *et al.* 2002), we selected 52 markers located on the Kasalath fragment of BIL-55 and performed genotype screening of Nipponbare, BIL-55, and Kasalath. Of these, 49 markers showed polymorphism between Nipponbare and BIL-55 or between Nipponbare and Kasalath.

QTL analysis using F_2 population

We used 139 F_2 plants derived from the cross between BIL-55 and Nipponbare for QTL analysis. We selected BIL-55 as a parent for the F_2 population, because it flowers as late as Kasalath under SD conditions, and harbors the Kasalath chromosome segment where the QTLs detected using BIL lines were located. A single seed was sown in commercial nursery soil (Minoru Industrial Co., Ltd., Okayama, Japan; basal fertilizer N:P:K = 0.3:0.45:0.3 g/L of soil) in a 5-cm-square pot, and plants were grown under SD conditions (10L/14D) in daylength treatment facilities in the field in Kyoto. Additional fertilizer (N:P:K = 0.2:0:0 g/L) was applied monthly until heading. All tillers except the main culm were removed. Addition to 49 SSR markers, we developed two CAPS markers using the polymorphisms between Nipponbare and BIL-55 based on genome sequences of QTL-seq: CAPS_02 (Forward primer: acaacattcacctgcttg gag, Reverse primer: tgacacaaattctctgtgagg, restricted by *Eco*RI) and CAPS_08 (Forward primer: caccgttggtggctcttttg, Reverse primer: tctgagcgccagtttctgag, restricted by *Eco*RI). To detect QTLs, we used marker regression analysis and composite interval mapping (CIM). CIM gave plots with peaks suggesting possible QTLs. To identify QTLs, we used R/qtl with 49 SSR markers and two CAPS markers. The R/qtl functions “makeqtl” and “fitqtl” were used to estimate the QTL effects. QTLs for F_2 population were considered significant at $P < 0.05$, with an LOD score calculated from 1000 permutations.

DNA extraction and bulking for QTL-seq

Leaf samples of 384 F_2 plants and the parents (Nipponbare and BIL-55) were collected and freeze-dried. DNA was extracted from the bulked leaves of each sample by the CTAB method. The quantity and quality of DNA were assessed by NanoDrop and Qubit spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the concentration was adjusted to 50 ng/ μ L. From the distribution of heading date among the population, we selected 10% earliest plants (divided into two sub-groups [12 and 27 plants], total 39 plants) and 10% latest plants (divided into four sub-groups [22, 6, 6, and 5 plants], total 39 plants) as the early-DTH groups and late-DTH groups, respectively. From each group, we extracted 1 μ g of DNA. We also prepared 1 μ g of DNA from 12 bulked plants each of Nipponbare and BIL-55.

DNA library construction and sequencing for QTL-seq

The genomic DNA was sheared to an average fragment

size of 300 bp in an Adaptive Focused Acoustics sonicator (Covaris, Inc., Woburn, MA, USA) and purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). DNA libraries were constructed using a NEBNext DNA Library Prep Master Mix Set for Illumina and NEBNext Multiplex Oligos for Illumina according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). In brief, the fragmented DNAs were end-repaired, dA-tailed, and ligated with the NEBNext adapter. Size selection was conducted using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions. The adapter-ligated DNAs were amplified by eight cycles of high-fidelity PCR with eight different index primers (index 2, 4, 5, 6, 7, 12, 16, and 19). The PCR products were cleaned up using AMPure XP magnetic beads. The library quality and concentration were assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The library concentrations were determined precisely by quantitative real-time PCR using a KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA).

All libraries were diluted to 10 nM and then mixed in equal amounts. After denaturation with 0.2 N NaOH, the final concentration of the library mixture was adjusted to 10 pM, including 1% PhiX library (Illumina, Inc., Woburn, MA, USA). Illumina sequencing using the paired-end read (2 $\times$ 102-bp) protocol was performed on a HiSeq 2500 platform (Illumina). Read data in FASTQ format were converted from raw base-call data in CASAVA 1.8 software (Illumina), and all read lengths were adjusted to 100 bp by trimming each end of one base. The read data were submitted to the DDBJ Read Archive (accession number DRA004492).

SNP detection and QTL-seq analysis

Raw reads were processed by Trimmomatic 0.30 software to remove adapter sequences and low quality nucleotides (Bolger *et al.* 2014). Only read pairs whose length were more than 32 bp were used for further analysis. Processed reads were mapped on the rice genome (IRGSP-1.0, Kawahara *et al.* 2013) by BWA 0.7.9a software (Li and Durbin 2009). Mapped reads were filtered by SAMtools 1.1 software (Li *et al.* 2009) (-q 30 -F 0x100 -f 0x2). Early and late samples were mapped and filtered separately by group. Then BAM files for the 2 early bulks were merged into 1 bulk, and those for the 4 late bulks were merged into another bulk. PCR duplication was removed by Picard Tools 1.94 software (<http://picard.sourceforge.net>). To improve mapping quality, local realignment was performed and SNPs were detected in GATK 3.2-2 software (McKenna *et al.* 2010) (--min_base_quality_score 20).

QTL-seq analysis was used to reveal a minimum set of Kasalath genomic regions for late heading date under SD conditions. For QTL-seq analysis, we followed Takagi *et al.* (2013). We used only sites at which Nipponbare and BIL-55 had homozygous SNPs with a read depth of >4 ; early bulks

had a read depth of 20–100; and late bulks had a read depth of 40–100. Then the SNP-index was calculated for each SNP position (Abe *et al.* 2012, Lu *et al.* 2014, Takagi *et al.* 2013). Sliding window analysis was performed with a 2 Mb window and 10 kb increments. The average SNP-index of the SNPs in the window was calculated and plotted. The SNP-index of individuals differentiating the early and late bulks was estimated and plotted. The Δ SNP-index (the SNP-index of the late bulk minus that of the early bulk) was calculated, and the average Δ SNP-index across a 1 Mb region was plotted using a 500 kb sliding window.

Candidate gene search

To find candidate genes responsible for late heading, we annotated detected SNPs and detectable small InDels in SnpEff v. 3.2 software (Cingolani *et al.* 2012) using Rice Annotation Project annotation data downloaded from the RAP-DB (http://rapdb.dna.affrc.go.jp/download/archive/irgsp1/IRGSP-1.0_representative_2015-03-31.tar.gz) (Sakai *et al.* 2013). The effects of mutations were categorized as high impact, moderate impact, low impact or modifier (Cingolani *et al.* 2012).

Results

Evaluation of heading date using CSSLs and NILs

Under SD conditions, DTH was 51 ± 1.7 days in Nipponbare and 91 ± 2.3 days in Kasalath (Fig. 1a). To detect QTLs for late heading, we grew CSSLs and their parents, Nipponbare and Kasalath, and measured their DTH. The differences in DTH of the CSSLs are shown in Fig. 1b. DTH in CSSLs was only ± 10 days relative to Nipponbare. The similarity to that in Nipponbare suggests that late heading under SD conditions was not regulated by one major gene from Kasalath.

NIL (*Hd1*) headed >10 days later than Nipponbare, and NIL (*Hd1*, *Hd2*) headed >25 days later than Nipponbare, but the other combinations headed earlier than NIL (*Hd1*) (Fig. 1b). These results indicate that *Hd1* and *Hd2* from Kasalath are partly but not wholly responsible for late heading.

Evaluation of heading date and QTL analysis using BILs

Since none of the CSSLs or NILs headed as late as Kasalath (Fig. 1b), we genetically analyzed BILs from crosses between Nipponbare and Kasalath to find QTL regions responsible for late heading. DTH under SD conditions among the 98 BILs ranged from 43 to 90 days (Fig. 1b). Most BILs headed later than Nipponbare, 39 of them significantly later ($P < 0.05$), of which five headed >30 days later than Nipponbare: BIL-51 (DTH = 84 days), BIL-55 (88 days), BIL-61 (83 days), BIL-78 (90 days), and BIL-96 (84 days) (Fig. 1b). Of these, BIL-55 and BIL-78 headed almost as late as Kasalath. We selected BIL-55 for further QTL analysis because only this line produced F_2 seeds.

First, we analyzed genotypes of known flowering genes. As NIL(*Hd1*, *Hd2*) headed >25 days later than Nipponbare, we performed marker analysis to detect *Hd1* (marker R2171) and *Hd2* (marker C728) in the BILs (Supplemental Fig. 1). BIL-55 had the Kasalath-type *Hd1* but not the Kasalath-type *Hd2* (Fig. 1c, Supplemental Fig. 1).

To eliminate the possibility of the existence of a small *Hd2* fragment from Kasalath in BIL-55, we genotyped the whole genome of plants by the Illumina GoldenGate assay. The SNPs around *Hd2* were the Nipponbare type in BIL-55, confirming that BIL-55 had no Kasalath-type *Hd2* fragment (Supplemental Table 1). Among all five late-heading BILs, all *Hd1* alleles came from Kasalath, and *Hd2* alleles came from Nipponbare except in BIL-51 and BIL-78 (Supplemental Fig. 1, Supplemental Table 1). These results indicate that the Kasalath-type *Hd1* (nonfunctional) is required for late heading under SD conditions, but the Kasalath-type *Hd2* is not necessary.

We also carried out QTL analysis using 98 BILs for rough mapping (Supplemental Fig. 2a, 2b). This analysis identified two QTLs on Chrs. 4 and 6 (Supplemental Fig. 2c). The QTL (marker R2171) on Chr. 6 was consistent with the *Hd1* locus, but that on Chr. 4 (marker R514) was not reported. The LOD scores of other peaks, on short arm of Chrs. 1 and 6 (*Hd3a* locus), and Chr. 12 were less than 9.68, indicating no significant.

Detection of QTLs associated with late heading in BIL-55

Analysis of CSSLs, NILs, and BILs indicated that multiple QTLs or genes are involved in DTH control under SD conditions and that not only *Hd1* but also novel QTLs might be involved. To detect novel QTLs, we developed an F_2 population from a cross between BIL-55 (Fig. 1c) and Nipponbare. We selected 49 SSR markers based on Kasalath fragments of BIL-55 and performed QTL analysis in 139 F_2 plants (Supplemental Fig. 3a, 3b). The analysis identified one significant peak on Chr. 4 (marker RM2441) and three on Chr. 6 (RM6003, RM6302, RM7193) ($P < 0.01$; Supplemental Fig. 3c, Table 1). The percentage of phenotypic variation explained (PVE) by individual QTLs ranged from 3.6% to 20.5%. Two QTLs on Chr. 6 (RM6003 and RM6302) were consistent with *Hd3a* and *Hd1*, respectively. *Hd1* had a positive additive effect, indicating that the Kasalath allele increased DTH, whereas *Hd3a* had a negative additive effect, indicating that the Nipponbare allele increased DTH (Table 1, Supplemental Fig. 4). These results confirm known genes. We identified two unreported QTLs: on Chr. 4 (RM2441), which we named *qDTH4.5*, and on Chr. 6 (RM7193), which we named *qDTH6.3*. Of these, the marker position of *qDTH4.5* (RM2441, position 28,047,569 bp of pseudomolecule IRGSP-1.0) was very close to the QTL on Chr. 4 in the BIL population (R514, position 30,291,703 bp of pseudomolecule IRGSP-1.0) (Supplemental Figs. 2, 3), suggesting that these QTLs are identical to each other. Both *qDTH4.5* and *qDTH6.3* showed positive additive effects, indicating that the Kasalath alleles increased DTH (Table 1,

Fig. 1. (A) Mean DTH of Kasalath (91 ± 2.3 days) and Nipponbare (NPB; recurrent parent; 51 ± 1.7 days) under 10L/14D SD conditions. (B) Differences in mean DTH between CSSLs, NILs, BILs, and Nipponbare (recurrent parent) under 10L/14D SD conditions. Bars denote the difference in the mean DTH of each line ($\pm$ standard deviations) from Nipponbare (NPB). Black bars denote difference between donor cultivar Kasalath (Kas) and Nipponbare. Line names are indicated below. Asterisks indicate $P < 0.01$ in t-test. (C) Graphical genotype of BIL-55, which was used for QTL analysis by SSR marker-based and QTL-seq under SD conditions. Black, homozygous for Kasalath; white, homozygous for Nipponbare; gray, heterozygous. Gray and black boxes on the short arm of Chrs. 4 and 10 indicate newly identified Kasalath homozygous region by the QTL-seq analysis and heterozygous region by the illumina GoldenGate assay, respectively.

Table 1. QTLs for heading date detected under SD conditions by composite interval mapping

QTL name	Chr.	Candidate gene	Nearest Marker ^a	Physical Position (bp) ^b	LOD ^c	AE ^{d,e}	PVE ^d
<i>qDTH4.5</i>	4	unknown	RM2441	28,020,850	10.8	2.7	11.6
<i>Hd3a</i>	6	<i>Hd3a</i>	RM6003	2,104,200	8.3	-1.7	8.5
<i>Hd1</i>	6	<i>Hd1</i>	RM6302	8,984,566	13.8	4.4	15.5
<i>qDTH6.3</i>	6	unknown	CAPS_08	18,167,849	3.3	4.0	3.3

^a Nearest markers denote the relevant markers that were closest to the peak of QTLs.
^b Location of pseudomolecule IRGSP-1.0 (RAP-DB:<http://rapdb.dna.affrc.go.jp/>).
^c The significant threshold of LOD value of DTH was 2.99 and 3.49 ($P < 0.05$ and 0.001), respectively.
^d AE: additive effect, PVE: percentage of total phenotypic variance explained in each QTL.
^e Minus values of additive effect indicate that ‘Kasalath’ alleles decrease DTH.

Supplemental Fig. 4).

In the above QTL analysis, the SSR markers in BIL-55 were positioned only in Kasalath regions determined by the Illumina GoldenGate assay. Therefore, if small Kasalath regions that were not detected by the assay contributed to the late heading, the SSR marker-based analysis would not detect them. To test this possibility, we performed QTL-seq analysis. Since QTL-seq is based on whole-genome re-sequencing, it can determine not only omissions (by SSR or GoldenGate assay), but also all polymorphisms within genes in candidate QTL regions. The distribution of DTH in the F₂ population from the cross between Nipponbare and BIL-55 is shown in Fig. 2; 10% of segregants with each of the two extreme phenotypes (early and late) were used. The

re-sequencing of parents yielded 34 million read pairs, representing ~90x of the IRGSP-1.0 rice genome (Supplemental Table 2). Mapping-based SNP detection using the IRGSP-1.0 rice genome showed SNP-dense regions (Supplemental Table 3). Most of them matched Kasalath regions determined by the Illumina GoldenGate assay (Supplemental Table 1, Fig. 1c), but we newly found a Kasalath homozygous region and heterozygous region in the telomeric region of the short arm of Chrs. 4 and 10 (Fig. 1c). We generated 121 million reads for early bulks and 229 million reads for late bulks (Supplemental Table 2), of which 77.4% and 74.3% were mapped on the reference genomes with high quality, covering 97.4% and 98.0% of the reference genome with a read depth of >4 (Supplemental

Fig. 2. Frequency distribution of DTH of 384 F₂ and parental plants under SD conditions. The DNAs of 39 early-heading plants (<40 days) and 39 late-heading plants (>53 days) were each bulked. The DNAs of 12 Nipponbare plants and 12 BIL-55 plants were also bulked.

Fig. 3. QTLs detected by QTL-seq analysis and SSR marker-based QTL analysis. (Top) Early- and late-heading bulks (Fig. 2) were analyzed by QTL-seq, using Nipponbare as the reference genome. Three peaks Δ SNP-index were identified on Chrs. 4 and 6. (Bottom) SSR marker-based QTL analysis of 139 F₂ plants were detected by 49 SSR and 2 CAPS markers in BIL-55, which were positioned only in Kasalath regions determined by the Illumina GoldenGate assay. LOD scores were obtained by marker regression analysis (blue) and composite interval mapping analysis (black). Gray rules indicate LOD thresholds (1000 permutations) at $P < 0.01$.

Fig. 5). Comparative sequence analyses of the parents identified 836,310 SNPs and InDels. After filtering by read depth, we selected 484,127 SNPs and InDels for QTL-seq analysis (Supplemental Table 3). Sliding window analysis of the Δ SNP-index showed QTL candidates in three genomic regions. Two QTLs detected by SSR marker analysis were distributed in a long region (9.5 Mbp: 9.0–18.5 Mb of pseudomolecule IRGSP-1.0) on Chr. 6 (Fig. 3, Supplemental Figs. 6–8). The other, on the short arm of Chr. 6, was detected as a significant reverse peak (Nipponbare allele delayed heading) of the Δ SNP-index value (Fig. 3). In addition, the QTL on Chr. 4 was also detected (as a mound

shape) by QTL-seq analysis (Fig. 3). Therefore, all statistically significant peaks of the Δ SNP-index value were consistent with QTLs detected by SSR marker analysis.

Characterization of detected SNPs in QTL regions

In an attempt to identify candidate genes for the novel QTLs from the whole-genome re-sequencing data, we confirmed SNPs for *Hd1* and *Hd3a*. We detected all reported polymorphisms (Kojima *et al.* 2001, Yano *et al.* 2000) in both genes (data not shown). Then we defined the physical position of *qDTH4.5* and *qDTH6.3* by integration of results derived from QTL analyses and the Δ SNP-index values of QTL-seq. *qDTH4.5* (Chr. 4) was narrowed down to 26.5–29.0 Mb of pseudomolecule IRGSP-1.0 at the top of the peak region, and *qDTH6.3* (Chr. 6) to 17.5–18.5 Mb (right-hand end of the flat peak; Supplemental Fig. 9). We compared the Kasalath and Nipponbare sequences and listed genes identified by SnpEff as “high impact”. We identified 29 genes (7 stop-gained, 4 stop-lost, 2 splice-site and 23 frame-shift mutation in 36 transcripts) in the *qDTH4.5* region and 11 genes (2 stop-gained, 3 stop-lost, 1 splice-site and 11 frame-shift mutation in 17 transcripts) in the *qDTH6.3* region with SNPs/InDels that led to defective function (Supplemental Table 4). Among these genes, there are no homologs to flowering time related genes so far identified in any plant species. In addition, we identified 226 missense mutations in 122 genes (substitutions that result in a functionally different amino acid) in the *qDTH4.5* region and 105 in 95 genes in the *qDTH6.3* region, respectively (Supplemental Table 5). In the *qDTH4.5* region, missense mutations were present in genes for Zinc finger B-box domain containing protein (Os04t0540200-01), WD40-repeat-domain-containing proteins (Os04t0555500-01, Os04t0555600-01, Os04t0564700-01), and similar to flowering locus D (Os04t0560300-01); and in the *qDTH6.3* region, they were present in genes for PHD CCAAT-binding-domain-containing protein (Os06t0498450-00). Although these genes share domains with known any flowering time genes in other species, no definitive functional mutation was found.

Discussion

We identified four genomic regions responsible for heading date on Chrs. 4 and 6 by SSR marker-based QTL analysis in a cross combination between Nipponbare and BIL55. These QTLs were confirmed by QTL-seq analysis followed by whole-genome re-sequencing. Most SNP-index values of the early-heading lines on Chr. 6 at 9.0–18.5 Mb were spread out at <0.5 (Supplemental Fig. 6), whereas those of the late-heading lines in the same region were >0.8 (Supplemental Fig. 7). In the *qDTH4.5* region, SNP-index values of the early-heading lines on Chr. 4 at 28 Mb (Supplemental Fig. 6) were <0.4, whereas those of the late-heading lines were mostly >0.4 (Supplemental Fig. 7). Of these, two on Chr. 6 were consistent with *Hd1* and *Hd3a*,

Fig. 4. QTL locations for heading date under SD and LD conditions based on the linkage map reported in Yano *et al.* (2000). The vertical open bars numbered 1 to 12 denote the 12 rice chromosomes. The names of the markers and their chromosomal positions are indicated to the left of each bar. *Hd1-18* were detected as QTL for heading date under SD or LD conditions. Black bars denote the QTL regions for heading date detected under SD conditions.

respectively. *Hd1* promotes heading under SD conditions (Yano *et al.* 2000), and *Hd3a* promotes it under SD and LD conditions (Kojima *et al.* 2002). Since the Kasalath allele of *Hd1* is defective and that of *Hd3a* is more effective on promoting flowering time than that of Nipponbare (Yano *et al.* 2000, Kojima *et al.* 2001), these results coincide with past reports. Other than *Hd1* and *Hd3a*, some genes or loci, *Ef4*, *Ef5* and *Ef7*, controlling heading date under SD conditions were identified (Kuhn *et al.* 2004, 2005, Saito *et al.* 2012). However, *qDTH4.5* and *qDTH6.3* were located on different chromosome from the *Ef4*, *Ef5* and *Ef7* loci. Therefore, we concluded that these two QTLs, *qDTH4.5* and *qDTH6.3*, are novel (Fig. 4). *qDTH4.5*, detected on Chr. 4 in the F_2 population, was very close to a QTL detected on Chr. 4 in the BIL population. Therefore, *qDTH4.5* might be identical to the QTL detected in the BIL population. *qDTH4.5* showed the 2nd highest genetic effect after *Hd1* (PVE = 12.5%) in F_2 analysis (Table 1). On the other hand, *qDTH6.3* was detected on Chr. 6 only in the F_2 population, by both SSR marker-based mapping and QTL-seq analysis. *qDTH6.3* had a lower genetic effect than *qDTH4.5* but a higher additive effect. However, since *qDTH6.3* linked to *Hd1*, it had possibilities that genetic effect of *qDTH6.3* was not precisely es-

timated. To estimate genetic effect and interaction of *qDTH6.3* with *Hd1* precisely, some cross populations among BILs such as BIL55 $\times$ BIL61 or BIL55 $\times$ BIL78, or between BILs and CSSLs such as BIL55 $\times$ SL39, are useful to simplify their genetic effects on heading date under SD condition. Because all of these lines harbor Kasalath allele at the *Hd1* locus, which is the most effective gene for late heading, we can eliminate its effect and define the effect of *qDTH6.3* and other QTLs. Nevertheless, genetic interaction between *qDTH6.3* and *Hd1* was revealed as a long peak of Δ SNP-index in the QTL-seq analysis (Supplemental Figs. 6–8). These results indicate that both Kasalath-type *qDTH6.3* and *Hd1* are required for late heading under SD conditions. In addition, since we used an F_2 population for the QTL-seq analysis, we should expect a large linkage block. Although *qDTH6.3* and *Hd1* are necessary to delay heading, CSSLs with Kasalath-type *qDTH6.3* and *Hd1* had early heading date, therefore implicating the existence of other genes contributing to delay of heading date (Supplemental Fig. 10). Moreover, the Kasalath-type *qDTH4.5* in addition to the Kasalath-type *qDTH6.3* and *Hd1* alleles was not enough to explain the late heading of the five late BILs. BIL-55 and BIL-78 had Kasalath fragments at *qDTH4.5*,

Hd1, and *qDTH6.3*, but BIL-51 did not have them at *qDTH4.5* or *qDTH6.3* (Supplemental Fig. 1). BIL-61 also had a Kasalath fragment at *qDTH4.5* but not at *qDTH6.3*. Therefore, other QTLs should be considered. One such candidate QTL is *Hd2*, as indicated by NIL (*Hd1*, *Hd2*) in Fig. 1b. The combination of Kasalath-type *Hd1* and *Hd2* clearly delayed heading (Fig. 1b). Because BIL-55 did not have a Kasalath fragment at *Hd2*, this region was not detected by SSR marker-based analysis and QTL-seq analysis. Further, Nipponbare allele at the *Hd3a* locus, which is detected as a QTL on the short arm of Chr. 6, showed a negative additive effect, increasing DTH (Table 1). On the other hand, Uga *et al.* (2003) reported *Hd15* as a QTL for heading date under SD conditions. Although a small peak near the *Hd15* on chromosome 12 was detected in the present study, this is not significant. One reason why *Hd15* was not detected in this study is that a specific genetic interaction might be necessary for *Hd15* to delay heading under SD conditions, because the single effect of *Hd15* is very weak (Fig. 1b). These results indicate that the heading under SD conditions as late as Kasalath must be determined by several combinations of many QTLs.

Through whole-genome re-sequencing, we discovered a Kasalath fragment on the short arm of chromosome 4 in BIL-55 that was not detected by the Illumina GoldenGate assay (Fig. 1c, Supplemental Table 1). Even though QTL-seq analysis detected no unique QTLs, the QTLs detected by QTL-seq analysis were consistent with those detected by SSR marker-based analysis. We could not separate two QTLs as two peaks of Δ SNP-index, which were identified to *Hd1* and *qDTH6.3* by SSR marker-based analysis, therefore it might be difficult to distinguish physically close QTLs by QTL-seq analysis in our F₂ population. In this case, we could narrow down two QTL regions as both ends of peak of Δ SNP-index because of the high-resolution QTL detection power of QTL-seq. Therefore, the combination of QTL-seq and rough mapping by SSR markers are powerful tool for identification of QTL regions.

Since the DTHs in the F₂ population under SD conditions were not normally distributed (Fig. 2), we considered that different QTLs from those favored by the bulks might delay heading under SD conditions. Therefore, we prepared separate DNA bulks for early- and late-heading lines. However, the QTL regions detected by using the separate DNA bulks were the same as the result by using combined bulk in each early- and late-heading lines (data not shown). To increase the resolution of QTLs, it is preferable to use recombinant inbred lines or to increase the population size.

QTL-seq analysis offers the benefit of identifying mutated genes (Takagi *et al.* 2013). In the QTL regions on Chrs. 4 and 6, there are 329 and 54 genes, respectively, of which 29 and 11 genes had high-impact mutation: frame shifts or nonsense mutations (Supplemental Table 4). None of the genes are homologous to any flowering time genes so far found in plant species. Late heading under SD conditions was observed in *indica* cultivars, not in almost all *japonica*.

This suggests the existence of unique flowering time (= heading date) genes at these loci in *indica* cultivars. Because we used Nipponbare as the reference genome sequence for functional annotation of SNPs, if *qDTH4.5* and *qDTH6.3* are unique in Kasalath or *indica* rice, this approach will not annotate SNP functions. To date, the pseudomolecule of Kasalath genome sequence has been reported (Sakai *et al.* 2014). Taken together with our data, it is informative to perform QTL-seq analysis and determinate causal genes. The QTL-seq approach using BILs with a *japonica* background will not only provide more information on QTLs, but will also reveal allele sequences in QTL regions. Our results are now available for breeding *indica* rice varieties cultivated in tropical and subtropical Asia by identifying causal QTL regions or genes.

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