Genetic analysis and fine mapping of *LH1* and *LH2*, a set of complementary genes controlling late heading in rice (*Oryza sativa* L.)

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Heading date in rice (*Oryza sativa* L.) is a critical agronomic trait with a complex inheritance. To investigate the genetic basis and mechanism of gene interaction in heading date, we conducted genetic analysis on segregation populations derived from crosses among the *indica* cultivars Bo B, Yuefeng B and Baoxuan 2. A set of dominant complementary genes controlling late heading, designated *LH1* and *LH2*, were detected by molecular marker mapping. Genetic analysis revealed that Baoxuan 2 contains both dominant genes, while Bo B and Yuefeng B each possess either *LH1* or *LH2*. Using larger populations with segregant ratios of 3 : 1, we fine-mapped *LH1* to a 63-kb region near the centromere of chromosome 7 flanked by markers RM5436 and RM8034, and *LH2* to a 177-kb region on the short arm of chromosome 8 between flanking markers Indel22468-3 and RM25. Some candidate genes were identified through sequencing of Bo B and Yuefeng B in these target regions. Our work provides a solid foundation for further study on gene interaction in heading date and has application in marker-assisted breeding of photosensitive hybrid rice in China.

Key Words: rice (*Oryza sativa* L.), heading date, fine mapping, complementary genes, gene interaction.

Introduction

Heading date (flowering time) is one of the most crucial agronomic traits in rice (*Oryza sativa* L.) for ecological adaptation to different cultivation areas and cropping seasons, and changes in heading date greatly impact rice yield. Therefore, control of heading date is a key objective in rice breeding. As such, heading date has been comprehensively studied, and hundreds of quantitative trait loci (QTLs) have been reported (<http://www.gramene.org/qtl>). In recent decades, with the rapid development of molecular marker-based mapping, great progress has been made in fine mapping and cloning of heading date QTLs (Li *et al.* 1995, Lin *et al.* 1996, 2003, Lin *et al.* 1998, Matsubara *et al.* 2008, Monna *et al.* 2002, Takahashi *et al.* 2001, Xiao *et al.* 1995, Xiong *et al.* 1999, Yamamoto *et al.* 2000, Yano *et al.* 1997). To date, nine QTLs for heading date, *Hd1*, *Hd3a*, *Hd6*, *Ehd1*, *Ehd2*, *Ehd3*, *Ghd7*, *Ghd8* and *Hd17* have been cloned using a map-based cloning strategy (Doi *et al.* 2004, Kojima *et al.* 2002, Matsubara *et al.* 2008, 2011, 2012, Takahashi *et al.* 2001, Xue *et al.* 2008, Yan *et al.* 2011, Yano *et al.* 2000).

Heading date is a complex trait which is affected not only by photoperiod sensitivity but also by basic vegetative growth and temperature sensitivity. Studies on heading date have focused on photoperiod sensitivity, the most important factor influencing flowering. Genetic analysis has been conducted under different day lengths, including long-day (LD), short-day (SD) and natural-field conditions (NF). Photoperiod control of heading date involves a complicated network of genes (Tsuji *et al.* 2011). Among the identified genes controlling heading date, a number of epistatic interactions have been detected, while the study of complementary genes postponing heading date has not been reported.

In this study, we found that the F₁ derived from two early heading *indica* maintainers, Bo B and Yuefeng B, had a significantly delayed heading date under natural-field conditions. Genetic analysis of the resultant F₂ population showed that the late heading phenotype was controlled by a pair of dominant complementary genes derived from each of the two parents. In addition, we discovered that an *indica* cultivar, Baoxuan 2, extremely late heading in natural-field conditions, possesses both of these dominant complementary genes. By analyzing the segregant populations derived from crosses between Bo B, Yuefeng B and Baoxuan 2, we fine mapped the two complementary genes, analyzed candidate genes in the mapping regions, and developed several usable DNA markers for marker-assisted breeding. These results

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Fig. 1. The pedigree of Bo B and Baoxuan 2.

provide a solid base for molecular cloning of the target genes and prompt further studies of digenic interaction in heading date of rice.

Materials and Methods

Experimental materials

Three rice cultivars were used in this study: Bo B and Yuefeng B, two excellent maintainer lines that are middle-season *indica* varieties from the middle-lower region of the Yangtze River and the south of China; and Baoxuan 2, a late heading *indica* variety from southern China that has partial consanguinity with wild rice (*Oryza rufipogon* Griff). The genetic pedigree of Bo B and Baoxuan 2 (Fig. 1) showed that they share part of the same heredity background. We produced crosses among Bo B, Yuefeng B and Baoxuan 2 and the resulting F_1 plants were self-pollinated to obtain F_2 populations (By F_2 : Bo B $\times$ Yuefeng B; Bb F_2 : Bo B $\times$ Baoxuan 2; Yb F_2 : Yuefeng B $\times$ Baoxuan 2). Recombinant plants selected from the three F_2 populations were developed to F_3 lines.

Field trials and phenotypic evaluation

The plants used in this study were grown under natural field conditions in the Taicang (31°N, 121°E), Jiangsu province, China, from mid-May to November. The mean day-length during the cultivation period was 13.5 h (<http://www.weather.com.cn>). All materials were planted according to well-established standards: 13.3 cm between plants within each row and 26.4 cm between rows; field management followed normal rice production conditions. For each plant of the mapping populations (F_2 and F_3), the number of days-to-heading (DTH) required from seeding to heading of the first panicle was scored. For each parental cultivar, 10 plants were scored and mean DTH was calculated.

DNA analysis

Genomic DNA from the mapping populations and three parental lines was extracted from the freshly harvested

leaves of 60-day-old plants by the cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980) with a minor modification. Polymerase chain reaction (PCR) amplification was conducted in 10 μ l reaction volumes containing 2 μ l (20 ng/ μ l) template DNA, 5 μ l Mix (Takara, Japan), 2 μ mol of each primer and 1 μ l H₂O. Amplification was performed using the following conditions: 5 min at 94°C, followed by 35 cycles (1 min at 94°C, 40 sec at 55°C, 1 min at 72°C), and a final extension at 72°C for 5 min. To detect polymorphisms, PCR products were electrophoresed on 8% polyacrylamide gel in Tris-borate EDTA buffer and the gel was silver stained after completion of the electrophoresis.

Bulked segregant analysis and linkage analysis

Bulked segregant analysis (BSA) (Michelmore *et al.* 1991) was performed to determine molecular markers linked to the target genes. Fifteen early-heading and fifteen late heading plants were selected from the F_3 segregant progenies to create the early-heading and late heading DNA bulks. The parental DNA and the two bulked samples were used for genotyping. A total of 181 polymorphic simple sequence repeat (SSR) markers, selected from the public database (Rice Genome Research Program 2007), were employed to construct the linkage map. Linkage analysis was performed using MAPMAKER 3.0 (Lander *et al.* 1987) based on genotypic and phenotypic data from segregant individuals of the F_3 population. Genetic distances were calculated by the Kosambi (1943) function.

To further analyze linkage, additional insertion/deletion (InDel) markers were developed by designing primers based on DNA sequence diversity between the *indica* cultivar '93-11' (<http://rise.genomics.org.cn>) and the *japonica* cultivar Nipponbare (<http://rgp.dna.affrc.go.jp/>). Single-nucleotide polymorphism (SNP) markers were subsequently exploited by sequencing chromosome regions of interest between the two parents. DNA markers used in this study are listed in Table 1.

Sequence analysis of candidate genes

Full genomes of Bo B and Yuefeng B were sequenced using Illumina/Solexa technology by BGI (Beijing Genome Institute, China). Candidate genes in the target region were also amplified from Bo B and Yuefeng B by PCR with LA-Taq (Takara, Japan). PCR products were purified using a PCR purification kit (Takara, Japan), introduced into the pGEM-T Easy Vector (Promega, USA), and transformed into *E. coli* strain DH5 α . The plasmid was sequenced with an ABI Analyzer. Sequence alignment was performed using DNAssist.

Expression analysis

Rice seedlings of 4-leaf date were transferred to a growth chamber to receive light treatment for 5 d. The treating conditions were set as following: 11 h dark and 13 h light with a temperature of 28°C. For quantitative RT-PCR, we isolated

Table 1. The polymerase chain reaction (PCR)-based molecular markers used in fine mapping

Marker	Type	Chr.	Forward primer (5'-3')	Reverse primer (5'-3')	Product size
RM21300	SSR	7	ATAGATTTTCGTTCCACCGATCC	GCTAATTATCGAAGCAACCACACC	226
RM3635	SSR	7	GAGAGACAGTGGAAGGGAAGACG	GTTCCCTCCCTCCTCCTAGTTCG	90
RM5436	SSR	7	TGAGCTGCACAAGACAGACAAGC	ACCATTTGAACAGGATGGACTGG	194
RM8034	SSR	7	ATTCCAACGGTCCAGTTCAGAGG	CACGCAGTGTGGTGTGGTAAGC	281
RM21323	SSR	7	ATTGCGATGTTTGGATGTACCG	AGCAACTCGTCAATGAACATGC	250
Indel7-1	InDel	7	CCAGGTGAGAGAGAAGACATGACC	CCATCAGTCACACAGATCGACTCC	280
Indel7-2	InDel	7	TATCAGGTGAAAAAAGGCGG	GGATGAGGGAAAAGGTAGGGG	160
RM22465	SSR	8	CAGAATAGCAAGCGAAGGGAAGG	CTTCTTCTCCCAGTGCACAAACC	81
RM25	SSR	8	GGAAAGAATGATCTTTTCATG	GAACATTGGTTTTGATGGTAG	265
RM1111	SSR	8	CCTATACCAAACCGAACC GAACC	CGCTGCCAATATCACCAAAGG	185
Indel22468-3	InDel	8	TTTGACCTGGCTTGCCCTCT	CACAACCTCAGCAGCTATA	211
Indel22468-4	InDel	8	ATGTCCTTTTGGTCCCTCCT	CTTCCCAGCTTTCCTTGTA	216
Snpl-1	SNP	8	ATTGTCCGATGGAGTGGTAG	TGTGCTTTAGATTGAGGTGG	356
Snpl-2	SNP	8	GATGTTATTGACCGTGTAC	CAAGAGCCAAAGAGTGTGC	455

total RNA from the leaves using an RNA extracting kit (TIANGEN, China) and treated with DNase I (Takala, Japan). cDNA was synthesized from 2 µg RNA using PrimeScript RT Master Mix (Takala, Japan). The quantitative analysis of gene expression was performed with SYBR Premix Ex Taq II (Takala, Japan) on an Applied Biosystems 7500 Real-time PCR System. The data were analyzed with the relative quantification method.

Results

Genetic segregation of DTH

Bo B and Yuefeng B are two excellent *indica* maintainer lines and used as breeding resources in the middle-lower region of the Yangtze River and the south of China. Their average days-to-heading (DTH) at Taicang (31°N, 121°E), Jiangsu province, China, are 68.2 ± 3.2 and 82.6 ± 4.3 (Fig. 2), respectively. Interestingly, the F_1 between Bo B and Yuefeng B has an average DTH of 136.2 ± 6.8 , a significant delay in growth period. In 2007, the resultant F_1 plants were self-pollinated to produce 1791 F_2 progenies that formed a large segregant population: 980 late heading individuals ($DTH \geq 115$ d) and 811 early heading individuals ($DTH < 115$ d) (Table 2 and Fig. 2A). The ratio of late heading progenies to early heading progenies was 9 : 7 ($\chi^2 = 0.86$, $P > 0.05$). In 2008, 15 individuals selected randomly from late heading F_2 progenies were planted to F_3 lines (150–200 plants for each family). Of the 15 F_3 lines, two populations showed a consistent late heading phenotype, six populations exhibited a segregant ratio of 3 : 1, and the remaining seven populations exhibited a segregant ratio of 9 : 7 (Table 2). In 2009, we analyzed the DTH of two F_2 populations derived from crosses between Baoxuan 2 ($DTH = 154.3 \pm 6.6$) and Bo B and between Baoxuan 2 and Yuefeng B and found that the segregant ratios of late heading to early heading were all 3 : 1 (BbF_2 : $\chi^2 = 1.98$, $P > 0.05$; YbF_2 : $\chi^2 = 0.03$, $P > 0.05$) (Table 2 and Fig. 2B, 2C). These results revealed that the late heading phenotype was controlled by a pair of dominant

complementary genes, which we named *LH1* and *LH2*. Genetic analysis also indicated that the parents Bo B and Yuefeng B each contain a homozygous dominant gene (*LH1* and *LH2*, respectively), while Baoxuan 2 contains both homozygous dominant genes.

Mapping of *LH1* and *LH2* using molecular markers

Six F_3 lines with segregant ratios of 3 : 1 in growth period were selected as primary populations for mapping the pair of genes controlling the late heading phenotype. In Line 1, fifteen late heading plants and fifteen early heading plants were selected to create late heading and early heading DNA bulks. A total of 597 SSR markers distributed over the 12 chromosomes (McCouch *et al.* 2002) were used to screen the parents (Bo B and Yuefeng B). Of these, 181 exhibited polymorphism and were applied to the late heading and early heading mixed bulks. Ten SSR markers showed diversity between the two bulked DNA samples and were employed to survey polymorphism in Line 1 individuals. Two out of the ten SSR markers, RM6338 and RM3755 (located on the short arm of chromosome 7), were found to be closely associated with the late heading time among these 198 F_3 plants. Linkage analysis with Mapmaker/Exp3.0 software revealed that the genetic distance between RM 6338 and RM3755 was 9.2 cM. This first late heading gene detected was named *LH1* (Fig. 3A).

Markers RM 6338 and RM3755 were applied to two bulked DNA samples from each of the remaining five F_3 lines, and found not to be associated with the late heading time in Lines 3 and 4. These results revealed that the late heading phenotypes in Lines 3 and 4 were controlled by another gene, dubbed *LH2*. Using the same procedure with Line 3, *LH2* was mapped to a 10.2 cM region on the short arm of chromosome 8 flanked by the SSR markers RM3572 and RM6208 (Fig. 4A). Genetic analysis found that the dominant *LH1* allele derived from parent Yuefeng B, and *LH2* derived from parent Bo B.

Fig. 2. Frequency distribution of days-to-heading in three F₂ populations. A. ByF₂ (Bo B × Yuefeng B), B. BbF₂ (Bo B × Baoxuan 2), C. YbF₂ (Yuefeng B × Baoxuan 2). Arrow indicates mean days-to-heading for Bo B, Yuefeng B, Baoxuan 2 and the corresponding F₁.

Fine mapping of *LH1*

To fine map *LH1* from Yuefeng B, the flanking markers RM 6338 and RM3755 were used to examine 732 individuals from four F₃ lines segregated around the *LH1* locus. A total of 18 recombinants were found. All recombinants were genotyped using five polymorphic SSR markers within the target interval (Fig. 3B). Recombination analysis revealed that *LH1* was located in a region between RM21300 and RM3635, with a map distance of 1.8 cM.

To further refine the position of *LH1*, an additional large

mapping population, BbF₂, containing 7411 F₂ plants derived from the cross between Baoxuan 2 and Bo B, was developed. Markers RM21300 and RM3635 were used to survey 1780 early heading plants in the BbF₂ segregation population. In total, 21 recombinants were identified between these two markers. Two additional Indel markers, Indel7-1 and Indel7-2, were designed based on the publicly available rice genome sequence and the likelihood of detecting diversity between Bo B and Baoxuan 2 was predicted by comparing sequences from Nipponbare (<http://rgp.dna.affrc.go.jp/>) and the *indica* cultivar '93-11' (<http://rise.genomics.org.cn>). A total of seven markers (Table 3) were used to genotype the 21 recombinants. The plants recombinant in the region between Indel7-1 and Indel7-2 are listed in Table 3. The recombinants BBE-42 and BBE-273 restricted *LH1* to the region downstream of RM8034, while recombinants BBE-98, BBE-489 and BBE-813 localized the gene to the region upstream of RM5436. Therefore, *LH1* was confined to the 63.7-kb region flanked by the SSR markers RM5436 and RM8034 (Fig. 3C).

Fine mapping of *LH2*

The same strategy was used to fine map the *LH2* gene originating from Bo B. A total of 31 recombinants were found between markers RM3572 and RM6208 among 363 individuals from two F₃ lines segregated around the *LH2* locus. Five polymorphic SSR markers within the target interval were used to screen the genotypes of the recombinants. Consequently, *LH2* was narrowed down to a region between RM1111 and RM22465 with a genetic distance of 2.4 cM (Fig. 4B).

To further locate *LH2*, another large mapping population, YbF₂, containing 3344 F₂ plants, was derived from the cross between Baoxuan 2 and Yuefeng B. 830 early heading individuals in the YbF₂ segregation population were genotyped using RM1111 and RM22465. In total, 48 recombinants were identified between these two markers. Four additional markers, snp1-1, snp1-2, Indel22468-3 and Indel22468-4 were developed. Indel markers were designed as mentioned above. SNP markers were designed by direct partial sequencing of certain chromosome regions between Baoxuan 2 and Yuefeng B. In total, eight markers were used to genotype the 48 recombinants, including four new, two flanking and two SSR markers within the interval (Table 4). Among these 48 recombinants, two individuals showed that the crossover occurred somewhere downstream of Indel22468-3, while five revealed that the crossover occurred somewhere upstream of RM25 (Table 4 and Fig. 4C). The plants recombinant in the region between snp1-2 and RM25 are listed in Table 4. Therefore, *LH2* was mapped between Indel22468-3 and RM25 with a physical distance of 177 kb (Fig. 4C).

Candidate genes in the target regions

Fine mapping placed *LH1* on chromosome 7 between markers RM5436 and RM8034. Based on the available sequence annotation database (<http://www.tigr.org>), there

Table 2. Genetic segregation analysis of days-to-heading in rice

Pedigree	Popul.	No. of plants			Percentage of late heading plants	χ^2 (3 : 1)	χ^2 (9 : 7)
		Early heading	Late heading	Total			
Bo B/Yuefeng B	F ₂	811	980	1791	54.7	392.2*	1.70NS
F ₃ -Line 1	F ₃	47	151	198	76.3	0.17 NS	32.2*
F ₃ -Line 2	F ₃	47	139	186	74.7	0.01 NS	28.5*
F ₃ -Line 3	F ₃	48	147	195	75.4	0.02 NS	29.0*
F ₃ -Line 4	F ₃	41	127	168	75.6	0.03 NS	25.5*
F ₃ -Line 5	F ₃	40	119	159	74.8	0.00 NS	22.3*
F ₃ -Line 6	F ₃	47	142	189	75.1	0.00 NS	27.3*
F ₃ -Line 7	F ₃	66	95	161	59.0	37.3*	0.49NS
F ₃ -Line 8	F ₃	80	96	176	54.5	39.2*	0.21NS
F ₃ -Line 9	F ₃	0	188	188	100.0	—	—
F ₃ -Line 10	F ₃	83	96	179	53.6	43.6*	0.50NS
F ₃ -Line 11	F ₃	72	100	172	58.1	26.1*	0.25NS
F ₃ -Line 12	F ₃	78	109	187	58.3	27.9*	0.32NS
F ₃ -Line 13	F ₃	0	164	164	100.0	—	—
F ₃ -Line 14	F ₃	72	101	173	58.4	25.5*	0.31NS
F ₃ -Line 15	F ₃	85	108	193	56.0	37.3*	0.01NS
Bo B/Baoxuan 2	F ₂	1780	5631	7411	75.9	1.98NS	42.6*
YuefengB/Baoxuan 2	F ₂	830	2514	3344	75.1	0.03NS	50.1*

NS: not significant at the 0.05 level, $P > 0.05$; * $P < 0.01$

Fig. 3. Fine mapping of *LH1*. A. Location of *LH1* on chromosome 7. B. High-resolution linkage map of *LH1* using ByF₃ population. cM, genetic distance between the SSR markers. C. *LH1* was located on a 63-kb region between markers RM8034 and RM5436. The number of recombinants between adjacent markers is indicated below the bar. D. The BAC AP003756 was annotated by TIGR (<http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice>).

were nine predicted genes located in the 63-kb target region (Fig. 3D), five of which had a full-length cDNA and were regarded as candidates for *LH1*. We sequenced the full genomes of Bo B and Yuefeng B and compared the nucleotide sequences of these five candidate genes. Three (LOC_

Fig. 4. Fine mapping of *LH2*. A. Location of *LH2* on chromosome 8. B. High-resolution linkage map of *LH2* using ByF₃ population. cM, genetic distance between the SSR markers. C. *LH2* was localized to a 177-kb region between markers Indel22468-3 and RM25. The number of recombinants between adjacent markers is shown below the bar.

Os7g15570, LOC_Os07g15600 and LOC_Os07g15640) showed differences between Bo B and Yuefeng B in their coding regions. The second gene identified in this study, *LH2*, was mapped between markers Indel 22468-3 and RM25 on chromosome 8. Thirty potential genes were identified in the 177-kb target region, fifteen of which had a full-length cDNA and were regarded as candidates for *LH2*. Polymorphisms between Bo B and Yuefeng B were found in

Table 3. Genotypes of key recombinants from BbF₂ population

Individual	Days-to-heading	Chr.	Markers						
			RM 21300	RM 21323	Indel 7-1	RM 8034	RM 5436	Indel 7-2	RM 3635
BBE-42	90 (EH)	7	H	H	H	H	B	B	B
BBE-98	75 (EH)	7	B	B	B	B	H	H	H
BBE-273	80 (EH)	7	H	H	H	H	B	B	B
BBE-299	85 (EH)	7	B	B	B	B	B	H	H
BBE-489	85 (EH)	7	B	B	B	B	H	H	H
BBE-552	90 (EH)	7	H	H	H	B	B	B	B
BBE-624	80 (EH)	7	B	B	B	B	B	H	H
BBE-813	65 (EH)	7	B	B	B	B	H	H	H
BBE-1214	70 (EH)	7	H	H	H	B	B	B	B
BBE-1533	70 (EH)	7	B	B	B	B	B	H	H

EH, early heading; B, Bo B homozygous genotype; H heterozygous genotype

Table 4. Genotypes of key recombinants from YbF₂ population

Individual	Days- to-heading	Chr.	Markers							
			RM 22465	Snp 1-1	Snp 1-2	Indel 22468-4	Indel 22468-3	RM 22475	RM 25	RM 1111
YBE4-8	68 (EH)	8	H	H	H	H	Y	Y	Y	Y
YBE15-8	85 (EH)	8	H	H	H	Y	Y	Y	Y	Y
YBE30-11	90 (EH)	8	Y	Y	Y	Y	Y	Y	H	H
YBE34-9	95 (EH)	8	Y	Y	Y	Y	Y	Y	H	H
YBE39-3	85 (EH)	8	H	H	H	H	H	Y	Y	Y
YBE45-11	70 (EH)	8	Y	Y	Y	Y	Y	Y	H	H
YBE48-8	90 (EH)	8	H	H	H	H	H	Y	Y	Y
YBE52-2	80 (EH)	8	Y	Y	Y	Y	Y	Y	H	H
YBE55-7	80 (EH)	8	Y	Y	Y	Y	Y	Y	H	H

EH, early heading plants; Y, Yuefeng B homozygous genotype; H heterozygous genotype

the coding regions of three candidates (LOC_Os08g07660, LOC_Os08g07730 and LOC_Os08g07810) according to the nucleotide sequence alignment results. Potential genes and alignment results are listed in Table 5.

Expression analysis

To investigate the relationship of *LH1* and *LH2* to genes associated with the circadian clock and those known to be involved in photoperiod flowering, we compared the expression patterns of six additional genes in Bo B, Yuefeng B and ByF₁ population under long day conditions. These six genes, including *OsGI*, *OsLhy* and *OsPRR*, were associated with the circadian clock, and *Hd1*, *Ehd1* and *Hd3a*, are known to have key roles in photoperiod-controlled heading (Doi *et al.* 2004, Kojima *et al.* 2002, Yano *et al.* 2000). The results showed that the expression level of these genes are slightly different between Bo B and Yuefeng B. However, the expression patterns in ByF₁ population appeared markedly vary. The expression of the clock-associated genes and *Hd1* is promoted, in contrast, the expression of *Ehd1* and *Hd3a* is suppressed in ByF₁ population (Fig. 5). According to the photoperiod flowering pathway (Tsuji *et al.* 2011), we speculate that the epistasis effect of genes *LH1* and *LH2* acts on the clock-related genes, which are upstream of *Hd1*, *Ehd1* and *Hd3a* in the photoperiod flowering pathway.

Discussion

Heading date has been extensively studied as one of the essential agronomic traits in rice. A total of 734 QTLs related to heading date have been reported to date (<http://www.gramene.org/qlt>). Epistatic interactions among these have also been observed (Gu and Foley 2007, Kojima *et al.* 2002, Komiya *et al.* 2009, Lin *et al.* 2003, Uwatoko *et al.* 2008, Yano *et al.* 2000, Yu *et al.* 2002) but have not lead to major phenotypic variation in heading date. In this study, we detected two new interacting genes, *LH1* and *LH2*. A single dominant gene did not cause variation in heading date but the two dominant genes together, regardless of homozygous or heterozygous types for each, significantly delayed heading date. This digenic interaction to produce a new phenotype is typical of the complementary gene model. This phenomenon was first discovered in pea flower colors by Bateson and Punnett (Bateson *et al.* 1906), and has also been reported in rice: Thakur and Roy (1975) found that the uneven kernel trait is controlled by two dominant complementary genes, *Una* and *Unb*, and clustered habit is controlled by *Cla* and *Clb*. Recently, many other dominant-complementary genes have been identified in rice: e.g. *Rhz2* and *Rhz3* controlling rhizomatousness (Thakur and Hille Ris Lambers 1989), and *Stva* and *Stvb* controlling RSV (resistance to

Table 5. Polymorphisms of candidate genes between Bo B and Yuefeng B

No.	Chr.	BAC	Gene name	NCBI locus	Full-length cDNA/EST	Gene annotation	SNP				InDel	
							Bo B	Yuefeng B	Bo B	In-TTC	Bo B	Yuefeng B
1	7	AP003756	LOC_Os07g15570	9054441-9056000	NM001065855	pentatricopeptide (PPR)	CCG/ACA/ CGG/AGA	CTG/ACG/ TGG/AAA	In-TTC	Del-TTC		
2	7	AP003756	LOC_Os07g15590	9063927-9066617	CT857039	expressed protein	-	-	-	-	-	-
3	7	AP003756	LOC_Os07g15600	9068117-9071382	AK067947	PRL1-interacting factor G	CTG	GTG	-	-	-	-
4	7	AP003756	LOC_Os07g15610	9079693-9080221	AK288525	hypothetical protein	-	-	-	-	-	-
5	7	AP003756	LOC_Os07g15640	9104013-9109947	NM001065857	selenium-binding protein-like protein, CRR4	ATT/CGG	ATC/AGG	-	-	-	-
1	8	AP005606	LOC_Os08g07490	4205886-4206807	AK240638	expressed protein	-	-	-	-	-	-
2	8	AP005606	LOC_Os08g07500	4210446-4211917	AK100902	DUF617 domain containing protein	-	-	-	-	-	-
3	8	AP005606	LOC_Os08g07540	4236013-4241588	AK101266	hemimethylated DNA binding domain containing protein	-	-	-	-	-	-
4	8	AP005606	LOC_Os08g07550	4241938-4242886	AK111274	hAT dimerisation domain-containing protein	-	-	-	-	-	-
5	8	AP005606	LOC_Os08g07600	4267286-4267978	AK111113	expressed protein	-	-	-	-	-	-
6	8	AP005164	LOC_Os08g07660	4289421-4290286	AK241700	expressed protein	-	-	In-TG	Del-TG		
7	8	AP005164	LOC_Os08g07690	4299556-4300473	AK062724	expressed protein	-	-	-	-	-	-
8	8	AP005164	LOC_Os08g07700	4302905-4303699	AK241762	AP2 domain containing protein	-	-	-	-	-	-
9	8	AP005164	LOC_Os08g07720	4315163-4317641	AK108243	transferase family protein	-	-	-	-	-	-
10	8	AP005164	LOC_Os08g07730	4323890-4328771	NM001067641	transferase family protein	GCG	GTG	In-GCG	Del-GCG		
11	8	AP005164	LOC_Os08g07740	4330976-4335183	AB693204	Hd5 gene for heading date 5	-	-	-	-	-	-
12	8	AP005164	LOC_Os08g07760	4343174-4349505	AK103038	BRASSINOSTEROID INSENSITIVE 1- associated receptor kinase1 precursor	-	-	-	-	-	-
13	8	AP005164	LOC_Os08g07774	4351810-4360461	AK071471	disease resistance protein RPM1	-	-	-	-	-	-
14	8	AP005164	LOC_Os08g07790	4362358-4364522	AK288674	CRS2-associated factor 2, mitochondrial precursor	-	-	-	-	-	-
15	8	AP005164	LOC_Os08g07810	4370025-4371933	AK73494	strictosidine synthase	GCG	GAG	-	-	-	-

Fig. 5. The expression of rice flowering genes in Bo B, Yuefeng B and ByF₁. qRT-PCR was performed with total RNA from leaves of 30-d-old plants under LD conditions. Samples were collected at the initiation of the light phase (ZT = 0). These experiments were repeated at least three times.

stripe virus) (Maeda *et al.* 2004). However, dominant complementary genes controlling heading date have never been reported. Here, the genes *LH1* and *LH2* identified in this study provide an example for further study of gene interactions and networks in the rice flowering pathway.

With the completion of the rice genome sequence project and advances in molecular marker-based mapping, many heading-date-related genes have been identified. Key heading date genes located on chromosome 7 are as follows (Fig. 6): *Hd2*, tightly linked with the RFLP marker C728 (Yano *et al.* 2000), *Hd4*, located between RFLP markers R46 and C39 (Lin *et al.* 2003), and *Ghd7*, located between SSR marker RM5436 and RFLP marker C39 (Xue *et al.* 2008). In the present study, we fine mapped *LH1* to a 63-kb region flanked by two SSR markers, RM5436 and RM8034, upstream of *Ghd7* and near the centromeric region of chromosome 7. Genetic prediction analysis was conducted using the JCVI database (<http://rice.plantbiology.msu.edu>) and nine potential genes were identified, of which five had full-length cDNA (Table 5). We sequenced the full genome of Bo B and Yuefeng B and compared their nucleotide sequences. Alignment results showed that three of these genes, LOC_Os7g15570, LOC_Os7g15600 and LOC_Os7g15640 have differences in their coding regions between Bo B and Yuefeng B. In LOC_Os7g15570, which encodes a pentatricopeptide repeat (PPR)-like protein, we found four amino acid changes and one serine deletion. LOC_Os7g15600 and LOC_Os7g15640, which are known to encode a putative PRL1-interacting factor G and a selenium-binding type protein, respectively, also showed several single-nucleotide substitutions between the two cultivars. Further study will be required to pinpoint the candidate gene for *LH1*.

We also found a second gene, *LH2*, which was located on chromosome 8 and subsequently limited to a 177-kb region through analysis of recombination events. In previous studies, there have been other reports of heading date genes on

chromosome 8 (Fig. 6) (Lin *et al.* 2003, Matsubara *et al.* 2011, Nonoue *et al.* 2008, Zhang *et al.* 2006, Zhou *et al.* 2001). One such gene, *Ghd8*, also named *Hd5* (Lin *et al.* 2003) and *DTH8* (Wei *et al.* 2010), is located in the interval of *LH2*. It is known to encode the OsHAP3 subunit of a CCAAT-box binding protein (HAP complex). To investigate whether *Ghd8* was the candidate gene, we sequenced and compared the nucleotide sequences of *Ghd8* in Bo B and Yuefeng B, yet no differences were found. We also sequenced and compared the other 14 candidates for *LH2*, three of which, LOC_Os08g07660, LOC_Os08g07730 and LOC_Os08g07810, showed polymorphisms in their coding regions between Bo B and Yuefeng B. These genes are known to encode a strictosidine synthase, a transferase family protein and an unknown protein, respectively. Our preliminary results have excluded *Ghd8* as a prospective gene but further study is required to confirm this. Previous studies have not shown that genes on chromosomes 7 and 8 have a complementary action to postpone heading date as we have reported here. Thus, we assume that *LH1* and *LH2* might be two novel genes. However, cloning and functional complementarily tests need to be conducted to further identify *LH1* and *LH2* and clarify the molecular mechanism that regulates heading date in rice and other plants.

Photoperiod control of heading date involves a complicated network of genes. We investigated the relationship of *LH1* and *LH2* to genes associated with the circadian clock and those known to be involved in photoperiod flowering. Expression analysis showed that the epistasis effects of *LH1* and *LH2* act on the clock-related genes, which are upstream of *Hd1*, *Ehd1* and *Hd3a* in the photoperiod flowering pathway. Gene cloning and transgenic test are needed to integrate the *LH1* and *LH2* genes into the gene network of flowering time in rice.

Heading date is a complex trait that is mainly affected by photoperiod sensitivity, basic vegetative growth and

Fig. 6. Linkage maps of chromosomes 7 and chromosomes 8 showing the locations of *LH1* and *LH2*. Genes in the parentheses on chromosome 8 means *Hd5*, *Ghd8* and *DTH8* are the same genes.

temperature sensitivity, among which photoperiod sensitivity is the most important factor influencing flowering. Most of the heading date related genes are sensitive to the photoperiod. In this study, we investigated the photoperiod sensitivity of Bo B, Yuefeng B, Baoxuan 2 and ByF₁ population under natural-field conditions. The DTH of Bo B and Yuefeng B vary only slightly between the Taicang (31°N, 121°E, Jiangsu Province, China, NLD: 13.5-h light on average) and Sanya (18°N, 109°E, Hainan Province, China, NSD: 11.5-h light on average), while the DTH of Baoxuan 2 and ByF₁ population increased more than 60 days under NLD conditions than NSD conditions. The results indicated that the epistasis of *LH1* and *LH2* increases the photoperiod sensitivity of rice.

Growth from sowing to heading is a complex period in rice comprising a vegetative growth phase and a reproductive phase. The vegetative growth duration is composed of a basic vegetative growth phase and a photoperiod-sensitive phase that is controlled by many genes and environmental factors such as temperature and day length. Many studies have shown that advanced heading date shortens the vegetative growth period of rice and results in a reduction of grain yield, while delayed heading provides the crop with sufficient vegetative growth to lead to better grain yield and/or biomass (e.g. Peng *et al.* 1999). In the rice-planted areas of

southern China, super hybrid rice has justifiably popularized planting only one crop per year. To make the most of light energy from the sun and improve rice yield, appropriately delaying heading date is a key selection criterion during rice breeding and production. As a prospective gene resource for late heading, *LH1* and *LH2*, a set of dominant complementary genes, could effectively promote the breeding of new hybrid rice combinations to achieve high yield, high quality and cost-saving potential.

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