Characterization of Epistatic Interaction of QTLs LH8 and EH3 Controlling Heading Date in Rice

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Heading date is a critical trait for adaptation of rice to different cultivation areas and cropping seasons. We evaluated the heading dates of 1,123 chromosome segments substitution lines (CSSLs) in the genetic background of an elite rice variety Huajingxian74 (HJX74). A CSSL with the substituted segments from Zhihu100 exhibited late heading under both natural long-day (NLD) and natural short-day (NSD) conditions, and the late heading phenotype was controlled by two novel epistatic loci on chromosome 8 and chromosome 3, respectively, termed LH8 and EH3. The function of EH3 was dependent on the LH8 genotype through epistatic interaction between EH3Zhihu100 and LH8Zhihu100 alleles. Genetic and molecular characterization revealed LH8 encodes a CCAAT-box-binding transcription factor with Heading date1 (Hd1)-binding activity and may delay flowering by repressing the expression of Early heading date1 (Ehd1).

Our work provides a solid foundation for further study on gene interaction in heading date and has application in breeding rice with greater adaptability.
much insight into the flowering control of rice, the underlying molecular mechanisms, especially the genetic interactions among these factors, are still not well understood.

The advantages of CSSLs in precise and detailed phenotype evaluation, minor QTL detection and validation of gene-gene and gene-environment interactions facilitated both genetic studies and rice improvement\(^\text{28–30}\). Therefore, we had constructed a library of 1,123 CSSLs in rice using HJX74, an elite indica variety from south China, as recipient and 24 accessions, including 14 indica and 10 japonica, collected worldwide as donors to understand the molecular mechanism of agronomic traits including heading date\(^\text{31,32}\). Each CSSL contains a very small number of well-characterized chromosome segments from one of the 24 donor varieties.

In this study, we found a CSSL with the substituted segments from Zihui100 exhibited late heading under both NLD and NSD conditions, and the late heading phenotype was controlled by two novel epistatic loci on chromosome 8 and chromosome 3, respectively, termed LH8 and EH3. Map-based cloning of LH8 revealed it encodes a CCAAT-box-binding transcription factor and is allelic to DTH8.

### Results

#### Identification of a CSSL with late heading

The procedure for the development of the CSSLs was summarized in Fig. 1. HJX74 (as the female parent) was crossed with one of the 24 donors (Zihui100 was taken as an example), and the F1 plants were backcrossed with HJX74 to develop the BC1F1 generation. Polymorphic SSR markers were used in the selection of the donor chromosomal segments. Using the same method, BC6F1 plants were obtained, and were self-crossed to produce BC6F2 lines in which the majority of genomic regions were homozygous for HJX74 alleles. Totally, 1,123 such CSSLs in the HJX74 genetic background were developed\(^\text{31,32}\). To further understand how flowering in rice was controlled, we evaluated the heading date of the 1,123 CSSLs under natural growth conditions. A CSSL, CSSL5, with substituted segments from Zihui100 exhibited late heading compared with the recipient HJX74 under both NLD (114.4 ± 1.4 d for CSSL5, 100.1 ± 0.5 d for HJX74) and NSD conditions (84.5 ± 1.6 d for CSSL5, 76.7 ± 1.4 d for HJX74) in 2009 (Fig. 2a and 2b).

To analyze the genetic basis for the late heading of CSSL5, we crossed CSSL5 with the recipient HJX74 (Fig. 1). 73 out of 326 F2 plants showed heading date later than that of CSSL5 (113.0 ± 1.3 d) under NLD conditions in 2010, and the whole population exhibited trimodal distribution of heading date fitting \(4:9:3\) segregation ratio \((\chi^2 = 4.67 < \chi^2_{0.05,2} = 5.99)\) (Fig. 2c). These results indicate that the late heading phenotype was controlled by two genes with negative epistasis.

#### Confirmation of the two genes by resequencing

To delimit the heading date genes on the substituted segments, CSSL5 and HJX74 were high-throughput genotyped by whole-genome resequencing, and an ultrahigh-quality physical map was constructed (Fig. 3). 11 substituted segments with a total length of 20.64 Mb derived from Zihui100 were found to be distributed over 6 chromosomes (Fig. 3 and Supplementary Table S1 online). Molecular markers were subsequently designed on the substituted segments, and were used to analyze their linkage with heading date phenotype of the F2 population. Two QTLs were identified on chromosome 3 and 8, respectively, designated as EH3 and LH8. EH3 was mapped in the 750 kb genomic region between the Id32 and Id33 markers and had the contributions to phenotypic variation by 14.0%, the allele from Zihui100 could shorten the heading date by 2.32d and the dominant effect was \(3.24\). LH8 was mapped in the 460 kb genomic region between the Id83 and Id82 markers and had the contributions to phenotypic variation by 65.3%. The allele from Zihui100 could delay the heading date by 6.62d and the dominant effect was 3.44 (Table 1).

### Figure 1 | Flowchart of the development of CSSLs and QTL analysis.

MAS: marker-assisted selection.
Zhihui100 allele at LH8 (i.e., increased days-to-heading (DTH)) was regardless of the allele status of EH3. In comparison, the effect of the Zhihui100 allele at EH3 (i.e., shortened DTH) was observed in two genotype classes, homozygous for the Zhihui100 allele at the LH8 locus and heterozygous, but not in the class homozygous for the HJX74 allele at the LH8 locus (Fig. 4a). Under NSD conditions, the Zhihui100 allele at LH8 was also observed to delay heading regardless of the allele status of EH3. However, the two-way ANOVA suggested the digenic interaction between EH3 and LH8 was not significant under NSD conditions (P = 0.883) (Fig. 4b).

Map-based cloning of LH8. To fine map the LH8 gene, we selected F2 plants with homozygous EH3 allele from HJX74 and heterozygous LH8 allele from Zhihui100 to generate F3 population (Fig. 1). F3 population showed bimodal distribution for DTH fitting 3:1 segregation ratio under both NLD and NSD conditions in 2012 ($\chi^2 = 0.04 < \chi^2_{0.05,1} = 3.84$ and $\chi^2 = 0.03 < \chi^2_{0.05,1} = 3.84$ for NLD and NSD, respectively), indicating the Zhihui100 allele at LH8 increased DTH in a dominant manner (Fig. 5a and 5b). 7 new markers were developed in the marker interval of Id83-Id82, and were used to analyze a total of 2,159 F3 plants. 6 recombinants were identified between markers RM22475 and RM25 (Fig. 5c). The self-pollinated progeny (F4 lines) of those 6 plants were used to determine the genotypes of LH8 (Fig. 1). The recombinants r2 and r5 restricted LH8 to the 28.3 kb genomic region between the Id87 and Id811 markers, including 3 putative genes, a transferase family protein (LOC_Os08g07730), a SERK-family receptor-like protein kinase (LOC_Os08g07760) and a CCAAT-box-binding transcription factor (LOC_Os08g07740) which had been reported to function in heading date control in rice (Fig. 5c). The sequencing of LH8 genomic regions from CSSL5 and HJX74 revealed two GGC insertion and a 1,116-bp deletion in LOC_Os08g07740 of HJX74 (Fig. 6a), resulting in a 2-glycine insertion in the middle and a big alternation in the C-terminal region of the translated protein, respectively (Fig. 6b). These results suggest that LOC_Os08g07740 corresponds to LH8 controlling the late heading in CSSL5.

Characterization of LH8 in rice. To examine whether the sequence mutations can alter the expression of LH8, we examined the expression levels of LH8 in CSSL5, HJX74 and NIL-LH8 (a line selected from LH8 F2 segregating population with homozygous EH3 allele from HJX74 and homozygous LH8 allele from Zhihui100) under NSD conditions in 2013. No significant difference in the LH8 expression levels could be detected among the three materials, suggesting that late heading did not result from the LH8 transcription. The expression levels of other heading date genes were also analyzed. For Hd1 and OsMADS50, no difference could be detected among the three materials under NSD conditions. The expression levels of tendon HL genes were less in NIL-LH8 than those in HJX74 under NSD conditions, indicating that LH8 may delay heading through suppressing the expressions of these floral transition activators. In contrast, the expression levels of Ehd1 and RTF1 were higher in CSSL5 than those in HJX74. The different expression levels of Ehd1 and RTF1 between CSSL5 and NIL-LH8 may result from the function of different EH3 alleles (Fig. 7a).

To study the involvement of the 2-glycine indel and the C-terminal region of the LH8 protein in its function, we tested the interaction between LH8 and H1d by yeast-two-hybrid method. Yeast cells that coexpressed the LH8[Zhihui100] bait and Hd1 prey fusion proteins were able to grow on media lacking histidine and adenine, indicating that these proteins can bind each other in yeast cells to promote the expression of the H1S3 and ADE2 reporter genes. However, the similar interaction could not be detected between LH8[HJX74] and Hd1, indicating the 2-glycine indel and the C-terminal interaction between EH3 and LH8 ($\chi^2 = 5.96e-06$). Orthogonal contrast test showed that AA (Additive by additive), but not AD (Additive by dominance), DA (Dominance by additive) or DD (Dominance by dominance) type of interaction was significant in the interaction of EH3 and LH8 ($\chi^2 < 0.0001$). The effect of the
region of LH8 might be important for its interaction with Hd1 (Fig. 7b).

**Discussion**

Until now, a total of 734 rice heading date QTLs have been reported (http://www.gramene.org/qtl), and some of them have been molecularly characterized. Besides understanding the biological function of single gene/QTL, clarification of genetic interactions among these genes/QTLs is also important. Actually, several examples of epistatic interactions among heading date genes/QTLs have been reported. By eliminating genetic background noises, CSSLs offer a practical solution to bridge the huge gap of knowledge between the
genotype and phenotype, and therefore, are especially useful in studying complicated traits including heading date. In this study, a novel epistatic interaction between \( \text{LH8} \) and \( \text{EH3} \) involved in flowering control in rice was detected. Under NLD conditions and \( \text{HJX74} \) genetic background, in plants homozygous for the \( \text{LH8} \) \( \text{HJX74} \) allele, difference in DTH between \( \text{EH3} \) \( \text{Zihui100} \) and \( \text{EH3} \) \( \text{HJX74} \) alleles was small (about 1 day), as shown in \( \text{HJX74} \) and NIL-\( \text{LH8} \) (a line selected from \( \text{LH8} \) \( \text{F2} \) segregating population with homozygous \( \text{LH8} \) allele from \( \text{HJX74} \) and homozygous \( \text{EH3} \) allele from \( \text{Zihui100} \) ) (Fig. 8a). A large effect of \( \text{EH3} \) \( \text{Zihui100} \) allele on heading promotion (about 7 days) occurred with the \( \text{LH8} \) \( \text{Zihui100} \) allele, indicating \( \text{EH3} \) \( \text{Zihui100} \) allele could function to shorten heading date only when \( \text{LH8} \) \( \text{Zihui100} \) allele was concurrently present, and therefore, the function of \( \text{EH3} \) was dependent on the \( \text{LH8} \) genotype through epistatic interaction between \( \text{EH3} \) \( \text{Zihui100} \) and \( \text{LH8} \) \( \text{Zihui100} \) alleles (Fig. 8a). This result suggests that \( \text{EH3} \) might function as a modifier of \( \text{LH8} \). Genetic interaction between \( \text{LH8} \) and \( \text{EH3} \) suggested that QTL pyramiding would be an effective method for the development of varieties with desirable heading dates under different growth conditions.

To facilitate the identification of the location of \( \text{LH8} \) and \( \text{EH3} \), the high-throughput genotyping technology was exploited. The recipient \( \text{HJX74} \) and CSSL5 were resequenced at the sequencing depth of 66- and 1-fold, respectively. Besides a large substituted segment from the short arm end to RM282 on chromosome 3 which was detected through traditional marker-assisted selection during CSSLs construction\(^3\), 10 additional substituted segments were identified distributing over 6 chromosomes, including some small introgressed segments that might be difficult to be detected by SSR markers (see Supplementary Table S1 online). Indeed, \( \text{LH8} \) was found on untargeted chromosomal region on chromosome 8. These results showed that the high-throughput genotyping method by resequencing has a higher accuracy than marker-assisted selection. Owning to the deep sequencing coverage of the recipient \( \text{HJX74} \), resequencing the CSSL5 with genome coverage as low as 1-fold was enough to rapidly validate and delimit the QTL, suggesting the efficiency of this mapping-by-sequencing method in discovering the genes responsible for quantitative trait loci. Our results presented a good example of combining the advanced material with uniform genetic background and high-throughput resequencing technology in economically accelerating gene identification.

The sequencing of \( \text{LH8} \) genomic regions from CSSL5 and \( \text{HJX74} \) revealed two GGC insertion and a 1,116-bp deletion in...
LOC_Os08g07740 of HJX74, resulting in a 2-glycine insertion in the middle and a big alternation in the C-terminal region of the translated protein, respectively (Fig. 6). Such sequence variations in the coding region of LOC_Os08g07740 between CSSL5 and HJX74 suggested that LOC_Os08g07740 might correspond to LH8 controlling the late heading in CSSL5 and NIL-LH8. LOC_Os08g07740 encodes a CCAAT-box-binding transcription factor which had been reported to strongly inhibit floral transition with names of DTH8, Gh8 and LHD121–23. Similar to DTH8 and Gh8, LH8 increased plant height and grain number per panicle under NLD conditions (Fig. 2 and Supplementary Table S2 online), indicating LH8 is allelic to DTH8, Gh8 and LHD1. Indeed, the two GGC insertion and one 1,116-bp deletion in the coding region of LOC_Os08g07740 of HJX74 had also been found in the ZS97 allele at Gh822. However, unlike DTH8 and LHD1 delaying flowering only under NLD/LD conditions21,23 and Gh8 functioning oppositely under SD and LD conditions21,22, LH8 delays the heading date under both NSD and NLD conditions. These differences in function may come from different gene-background interactions or different growth conditions. Therefore, genes controlling important agronomic traits are necessary to be further studied in different genetic backgrounds and under different natural field conditions to give a full understanding of its effects and to offer practical instructions in rice breeding.

Expression analysis revealed the transcripts of several flowering promoting factors including Ehd1, Hd3a and RFT1 were fewer in NIL-LH8 than those in HJX74 under NSD conditions, suggesting LH8 might delay flowering by down-regulating the expression of Ehd1, Hd3a and RFT1 (Fig. 8b). The expression levels of Hd1 and OsMADS50 showed no significant differences among HJX74, CSSL5 and NIL-LH8 under NSD conditions, suggesting they might function upstream of LH8 or parallel to LH8. Furthermore, no significant difference of LH8 expression were found among HJX74, CSSL5 and NIL-LH8 under NSD conditions, indicating the reason for the change of heading date was not caused by LH8 expression, but by nucleotide changes of the coding sequence. The 6-bp and 1,116-bp indels in the coding region of LH8 resulted in a big sequence alternation of the translated protein, especially in the C-terminal region. The protein encoded by LH8 is a putative HAP3 subunit of the HAP complex, which binds to the CCAAT box, a cis-acting element present in approximately 25% of eukaryotic gene promoters38,39. HAP proteins always form a HAP2/HAP3/HAP5 trimeric complex with DNA binding activity in mammals40,41. In Arabidopsis, CO could replace AtHAP2 in the HAP complex to form a trimeric complex, CO/AtHAP3/AtHAP5, as the CCT domain of CO and the DNA-binding domain of AtHAP2 are similar to each other42. In LD conditions, overexpression of AtHAP2 or AtHAP3 could delay flowering by impairing formation of the CO/AtHAP3/AtHAP5 complex, leading to decreased expression of FT42. As a homologue of CO, Hdl could be able to replace OsHAP2 in the HAP complex in rice. Our yeast-two-hybrid results revealed that Hd1_HDL4 could indeed interact with LH8_HDL4, but not with LH8_HDL4, indicating the sequence difference, especially in the C-terminal region, between LH8_HDL4 and LH8_HDL4 might be important for its interaction with Hdl_HDL4, and therefore for the regulation of rice heading (Fig. 8b). The domains responsible for the interaction between LH8_HDL4 and Hdl_HDL4 needed to be further investigated. However, the interaction between LH8_HDL4 and Hdl_HDL4 (Hdl from Nipponbare) could not be detected (data not shown), in consistent with the previous study that effects of Gh8 depend on the genetic background22. Consequently, whether the HAP complex could be formed or not was determined by the different subunits presented in different genetic backgrounds, possibly resulting in varied functions in flowering control.

Until now, 4 genes (Ehd4, OsMADS50, OsDof12, OsPhyB) on the short arm of chromosome 3 had been identified to be involved in flowering control in rice43,44. In our study, LH8 was mapped in the 750 kb genomic region between the Id32 and Id33 markers with 123 putative ORFs inside, including OsMADS50, OsMADS50, an ortholog of Arabidopsis SUPPRESSOR OF OVEREXPRESSISON OF CONSTANS1 (SOC1), functions upstream of Ehd1 to indirectly

**Figure 6 | Sequence comparision of LH8 from HJX74 and CSSL5.** (a). Structure of LH8 in HJX74 and CSSL5. Black and gray regions represent the ORF and UTR regions of LH8, respectively. The dotted line represents the 1,116 bp deletion in HJX74. (b). Protein alignment of LH8 from the predicted protein of HJX74 and CSSL5 using the EMBL software ClustalW2 multiple sequence alignment tool.
whether EH3 is allelic to OsMADS50 remains to be investigated. Further fine mapping and molecular identification of EH3 will be necessary to provide molecular evidence for the epistatic interaction between LH8 and EH3 in regulating heading date in rice.

Methods

Plant materials and growth conditions. CSSL5 is one of a set of 1,123 chromosome segment substitution lines, which were developed from backcross progenies (BC3:F2) derived from a cross between an elite indica variety from south china, HJX74, as the recurrent parent and another indica variety Zhihu100 as the donor parent. Plants were grown under natural short-day (from July to November) and natural long-day (from March to July) conditions in a paddy field in Guangzhou (23°07’N, 113°15’E), China. Heading date was defined as the time when the first panicle emerged.

High-throughput genotyping using whole-genome resequencing. DNA was extracted from 100 mg fresh rice leaves using the DNeasy Plant Mini Kit (QIAGEN Sciences). DNA was quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific), and about 5 μg of DNA was used for preparation of libraries for Illumina sequencing according to the protocol for the Paired-End DNA Sample Prep kit (Illumina). The libraries were used for cluster generation on a flow cell and sequenced for 76 cycles on an Illumina Genome Analyzer IIx. Base calling and filtering of low-quality bases were done using sequence control software real-time analysis, Base calling (BCL) converter and the GERALD module (illumina).

DNA extraction and PCR amplification. Fresh leaves were collected at the seedling stage and then ground in liquid nitrogen. Microquantities of DNA were extracted from fresh leaves of each individual using a previously reported method45. Amplification was carried out on the program for the initial denaturing step with 94°C for 3 min, followed by 35 cycles for 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, with a final extension at 72°C for 5 min. PCR products were separated on 6% non-denaturing polyacrylamide gel and detected using the silver staining method46.

Figure 8 | Schematic representation of the LH8 and EH3 mediated flowering pathway under natural growth conditions. (a). Epistatic model of rice heading controlled by LH8 and EH3. (b). A proposed model for the flowering pathway controlled by LH8 and EH3 in rice under natural growth conditions. LH8 could down-regulate Ehd1, which could act on Hd3a and RFT1, thus delaying flowering in rice under natural growth conditions. EH3 might function as a modifier of LH8 to shorten heading date only when specific LH8 background was present. The interaction between LH8 and Hd1 depended on different alleles presented in different genetic backgrounds, possibly resulting in varied functions in flowering control.
RNA Extraction and QRT-PCR. Total RNA from 30d-old leaves were isolated using TRIZOL reagent (Invitrogen) following the manufacturer’s instruction. First-strand cDNA was reverse transcribed from DNasel-treated RNA with oligo-dT as the primer using ReverTra Ace kit (Toyobo). Gene expression was measured by QRT-PCR using the ABI 7500 system (Life technologies). The QRT-PCR was carried out in a total volume of 20 μl containing 1X SYBR Green Master Mix (Life technologies). We normalized the expression levels by using UBC gene as internal control. Each set of experiments was repeated three times, and the relative standard curve quantification method was used to evaluate quantitative variation. The QRT-PCR procedure was conducted at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The QRT-PCR primers were listed in Supplemental Table S3 online.

Yeast two-hybrid assay. The cDNA fragments encoding the entire putative LHS and Hdl proteins were PCR-amplified, verified by sequencing and cloned into NdeI–EcoRI restriction sites of the MATCHMAKER two-hybrid prey vector pGADT7 or bait vector pGBK7 (Clontech), respectively. The bait and prey constructs were co-transformed into the yeast strain AH109, and the transformed cells were plated on synthetic defined medium containing all essential amino acids except leucine and tryptophan (SD/-Leu/-Trp) medium and incubated at 30°C for 3 d and then diluted and applied onto SD/-Leu/-Trp/-His/-Ade medium and SD/-Leu/-Trp medium (as loading control) and cultured at 30°C for 2–3 days. The interaction between HY1L and SE was used as a positive control. The primers used in vector construction were listed in Supplemental Table S3 online.

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Author Contributions

B.X, R.L, X.H, and Y.Y contributed equally to this work. B.X, R.L, and X.H. conceived and designed the experiments; B.X, R.L, A.A, and X.H. performed the experiments; B.X and R.L. analyzed the data; B.X and R.L. contributed reagents/materials/analysis tools; B.X, R.L, A.A, X.H and Y.Y. wrote the paper.
Author contributions
Z.Q.L. and G.Q.Z. designed the experiments. Z.Q.L. wrote the manuscript. J.B.C. and M.Q. performed QTL analysis. J.B.C. and X.Y.L. performed map-based cloning. C.C. and H.T.Z. performed sequencing analysis. Y.H.W. performed real-time RT-PCR. R.Z.Z. and X.L.F. performed yeast-two-hybrid analysis. All authors commented on the manuscript.

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