Natural Variation of OsHd8 Regulates Heading Date in Rice

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Abstract: Heading date, as one of the most important agronomic traits, is a fundamental factor determining crop yield. Although diverse genes related to heading date have already been reported in rice, the key gene that regulates heading date is still poorly understood. Here, we identified a heading date regulator, heading date 8 (OsHd8), which promoted the heading date under long-day conditions and encoded a putative HAP3 subunit of the CCAAT-box-binding transcription factor. It is localized in the nucleus and expressed in various tissues. Sequence analysis revealed that there were four SNPs and one InDel in the promoter region of OsHd8, which was involved in the regulation of some floral regulators including GHD7.1, SDG718, OsGI and HDT1. Further evolutionary analysis showed that OsHd8 presents divergence between indica and japonica, showing natural selection during the domestication of cultivated rice. These results indicate that OsHd8 plays an important role in the regulation of heading date, and may be an important target for rice breeding programs.

Keywords: heading date; Heading date 8 (OsHd8); promoter; natural variation; rice

1. Introduction

Flowering is an important process in plant transition from vegetative to reproductive growth [1,2]. Rice flowering is often affected by the external environment, including light, temperature, and nutritional conditions [3]. Appropriate flowering time is not only beneficial to the reproductive development of rice but also affects the yield of rice [4]. To date, many flowering-related genes have been identified in plants [5–10]. Chd7 encodes a protein with a CCT (CO, CO-LIKE and TIMING OF CAB1) domain that delays the heading date, increases the plant height and promotes panicle size development [5]. In Arabidopsis thaliana, CO (CONSTANS), as a key factor in the photoperiod pathway, promotes flowering under long-day (LD) conditions [11]. FT (FLOWERING LOCUS T) is a member of the PEBP gene family that shares homology with RAF kinase inhibitor proteins (RKIPs); these are activated by CO to regulate flowering [12–14]. Hd1 and Hd3a, a rice ortholog of the Arabidopsis CO and FT gene, respectively, have been identified to promote heading under short day (SD) conditions [6,9]. However, the regulation of Hd1 and Hd3a in rice is different from that in Arabidopsis thaliana. PhyB (phytochromes B) is involved in the post-translation regulation of Hd1 to regulate Hd3a expression, while overexpression of Hd1 inhibits Hd3a expression and delays flowering depending on phyB under SD conditions [15]. RFT1 (RICE FLOWERING LOCUS T 1), encoding the mobile flowering signal, is the closest homolog of Hd3a. In Hd3a-RNAi transgenic plants, the RFT1 gene is activated to promote flowering under SD conditions [10,16]. Ehd1 (Early heading date 1) encodes a B-type response regulator, which can promote the flowering of rice under SD conditions by regulating expression of the FT-like gene [8]. Ehd1, as a unique
floral activation pathway in rice, regulates the heading date by the protein complex of Ghd7-Hd1 and OsREI-OsRIP1 [17–19]. PPS (Peter Pan syndrome) is a homolog of the photomorphogenic gene COP1 (CONSTITUTIVE PHOTOMORPHOGENIC1) in Arabidopsis thaliana and promotes flowering by regulating the GA biosynthesis and suppressing the miR156/miR172 expression [8]. In addition, MADS-box genes have been shown to play a vital role in the flowering time, of which, OsMADS50 is an important flowering activator, controlling various downstream floral regulators in rice, including OsMADS1, OsMADS4, OsMADS15, OsMADS18 and Hd3a [20–23].

The HAP (heterotrimeric heme activator protein) family, a class of CCAAT box factor (CBF) or nuclear factor Y (NF-Y), have been identified as important regulators in rice [24]. The HAP complex consists of three subunits, namely HAP2 (NF-YA; CBF-B), HAP3 (NF-YB; CBF-A) and HAP5 (NF-YC; CBF-C) [25,26]. A total of 10 HAP2 (OsHAP2A–J) genes, 11 HAP3 (OsHAP3A–K) genes and 7 HAP5 (OsHAP5A–G) genes have been identified in rice, and recent studies revealed that HAP gene members play a vital role in the rice heading date [25]. HAP5B and HAP5D, as flowering inhibitory factors, directly interact with HAP3D, HAP3F and HAP3H protein to regulate the photoperiodic flowering response of rice under LD [27]. DHDI (DELAYED HEADING DATE1) is involved in the flowering development through binding to their target HAP5 family genes HAP5C and HAP5D [28]. OsHAP1 (Heme Activator Protein like 1), as a flowering repressor, can physically interact with the DTH8/HAP3H and Hd1, and repress the heading date in rice [4].

Transcriptional regulation is largely controlled through gene promoters and their contributing cis-acting elements (CREs) [29]. The diversity of cis-regulatory elements including auxin response elements (AuxREs), the abscisic acid response element (ABRE), A-box (TACGTA), C-box (GACGTC), and G-box (CACGTG) are associated with auxin response in Arabidopsis thaliana [30]. Introducing new or disrupting existing upstream CREs, including single-nucleotide polymorphisms (SNPs), segmental deletions, insertion of transposable elements, and copy number variations, often leads to changes in many agronomic characters, resulting in crop improvement [31–34]. 3-bp InDel in the promoter region of Sl-ALMT9 (Al-ACtivated MALATE TRANSPORTER9) disrupts a W-box binding site, which prevents binding of the transcription repressor WRKY42, and promotes the accumulation of malic acid in fruits [35]. OsREM20 (Oryza sativa REPRODUCTIVE MERISTEM 20) encodes a B3 domain transcription factor and controls the grain number per panicle in rice by affecting the binding efficiency of OsMADS34 to the CArG box in the promoter [33].

In this study, we identified an early-flowering gene OsHd8 in the early-flowering rice JiaHong2B (JH2B) through map-based cloning, which encodes a putative HAP3 subunit of the CCAAT-box-binding transcription factor, and regulates the expression of OsGI, OsSDG718, OsHDT1 and OsGHD7.1 to promote rice heading under long-day conditions.

2. Materials and Methods

2.1. Plant Materials and Cultivation

To clone OsHd8, a F2 population of 4500 individuals was generated from the cross of 1880/JH2B. A set of germplasms, including 101 rice varieties (59 indica (IND), 9 temperate japonica (TEJ), 9 tropical japonica (TRJ), 12 aus (AUS), 2 aromatic (ARO) and 10 admix (ADM)), were used for genotyping of OsHd8 (Supplementary Table S1). All the rice plants were grown in the ErZhou experimental field (30°40’ N, 114°88’ E) of Wuhan University.

2.2. Heading Date Investigation Map-Based Cloning

The 1880, JH2B two parents and F2 population were investigated for the heading date. Each plant recorded the heading date when the rice plant began to head with a single spike. A total of 606 F2 plants with extreme phenotypes, which defined as the first 15% of heading in the whole F2 population, derived from the cross of 1880/JH2B were used for mapping of OsHd8. Firstly, it was primarily located in the 3.3–6.53 Mb region of the chromosome 8 by BSA-Seq (bulked segregant sequencing), and then we used the F2 population of the target
QTL and mapped OsHd8 between the marker SNP7730 and SSR-1 (Supplementary Table S2) using a chromosome fragment substitution analysis.

2.3. BSA-Seq and Analysis of the Seq-BSA Data

For the BSA-seq, two DNA pools were developed by selecting the extreme early heading date plants and extreme late heading date plants from the F2 population. The extreme phenotype of the early growth period was defined as the first 15% of heading in the whole F2 population, and the extreme phenotype of the late growth period was defined as the last 15% of the heading in the whole F2 population. The early heading date pool (Z-pool) was made by mixing equal amounts of DNA from 50 extreme early heading date plants, and the late heading date pool (W-pool) was made by mixing equal amounts of DNA from 50 extreme late heading date plants. The DNA isolated from the two DNA pools were prepared for BSA sequencing.

Libraries for all the DNA pools were prepared according to the Illumina TruSeq Library Construction Kit. The DNA libraries were sequenced on Illumina Hiseq Xten PE15 (Illumina Inc., San Diego, CA, USA). The short reads from the two DNA pools were aligned to a Nipponbare reference genome (MSU Rice Genome Annotation Project Release 7) using the BWA software [36]. Reads of the Z-pool and W-pool were separately aligned to a Nipponbare reference genome and consensus sequence reads to call SNPs with the SAM tools software [36]. The SNP loci between the test samples and reference genome were obtained using the GATK software [37]. The Euclidean distance (ED) and SNP-index were calculated to identify the candidate regions of the genome associated with the heading date [38].

2.4. RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR) Analysis

Total RNAs were extracted from young rice leaves of 3-week-old seedlings using the TRIzol Reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was used for synthesizing the cDNA with a reverse transcription kit (Vazyme, Nanjing, China). Quantitative reverse transcriptase (qRT-PCR) was performed on a Roche LightCycler® 480II instrument using the Hieff qPCR SYBR Green Master Mix (No Rox) following the manufacturer’s instructions (Yeasen, Shanghai, China). The actin gene was used as the internal control. All assays were performed with three biological replicates and the relative expression level was analyzed with the $2^{-\Delta\Delta CT}$ method [39].

2.5. Vector Constructions and Plant Transformation

In order to construct a complementary vector, the upstream 2 kb promoter region of the ATG, gene coding region, and downstream 1 kb region of the OsHd8 gene, were amplified from the genomic DNA of 1880 and then constructed into the complementary vector pCAMBIA1301. To prepare the construction of the Hd8 overexpression vector, the CDS of OsHd8 was amplified from 1880 and was introduced into the vector pCAMBIA1301-Ubi. All the constructed vector plasmids were transformed into an Agrobacterium tumefaciens strain EHA105 and transferred by Agrobacterium-mediated transformation into JH2B.

2.6. Dual Luciferase (LUC) Analysis

In order to construct a complementary vector, the upstream 2 kb promoter region of the ATG, gene coding region, and downstream 1 kb region of the OsHd8 gene, were amplified from the genomic DNA of 1880 and then constructed into the complementary vector pCAMBIA1301. To prepare the construction of the Hd8 overexpression vector, the CDS of OsHd8 was amplified from 1880 and was introduced into the vector pCAMBIA1301-Ubi. All the constructed vector plasmids were transformed into an Agrobacterium tumefaciens strain EHA105 and transferred by Agrobacterium-mediated transformation into JH2B.
2.7. Evolutionary Analysis

The genome sequences of 3024 cultivars and 32 wild rice accessions were obtained from the Rice Functional Genomics and Breeding Database (RFGB, http://www.rmbreeding.cn/Snp3k, accessed on 20 April 2022) and OryzaGenome (http://viewer.shigen.info/oryzagenome/, accessed on 22 April 2022) [40,41]. The geographic information of cultivated rice populations was obtained from the MKBASE (http://www.mbkbase.org/rice/germplasm, accessed on 28 April 2022) and marked on the map using R software. The PopGenome package in the R software was used to calculate the parameters of genetic divergence for OsHd8 and its flanking regions between indica and japonica subspecies, including haplotype and nucleotide FST, Nei’s GST, Hudson’s GST and HST [42]. A phylogenetic tree of OsHd8 was constructed using the UPGMA method with MEGA7.0 [43], and a haplotype network was constructed using the pegas package in the R software [44].

2.8. Statistical Analysis

All the assays were performed on three biological replicates. The data were analyzed using the GraphPad Prism 9 software (https://www.graphpad.com/, accessed on 28 January 2022) and the means were compared by Student’s t-test, the * , ** and *** mean p < 0.05, 0.01 and 0.001, respectively. The primers used for genetic mapping, vector construction, PCR and qRT-PCR analysis were all listed in Supplementary Table S4.

3. Results

3.1. Genetic Analysis and Mapping of OsHd8 for Heading Date

In the breeding practice, we found that the heading date of the O. longistaminata introgression line 1880 and an early flowering variety JiaHong2B (JH2B), was about 92 days and 65 days (Figure 1A,B), respectively, showing a great difference. To explore the genetic basis for the heading date in 1880, genetic linkage analysis of 1147 F2 individuals derived from the cross of 1880/JH2B displayed a continuous distribution with an apparent valley bottom between 62 and 92 days (Figure 1C). These plants were then used for the short and long heading date pools. The two pools were then subjected to whole-genome sequencing up to >121 × coverage, and 835,204 high-quality single-nucleotide polymorphisms (SNPs) were identified. SNP-index analysis showed that there was only one obvious single peak on the short arm 3.3~6.53 Mb of chromosome 8 (Supplementary Figure S1), meaning that the candidate gene controlling heading date is possibly located in this region, and named as Hd8.

A total of 660 plants with extreme phenotypes were then selected from a 4500 F2 population of 1880/JH2B cross and were used for fine mapping; the OsHd8 successfully narrowed the locus to a 31.8 kb region between the marker SNP7730 and SSR-1 (Figure 2A). According to the information from the RGAP (Rice Genome Annotation Project), four predicted genes were present in this region, namely, LOC_Os08g07730, LOC_Os08g07740, LOC_Os08g07760, and LOC_Os08g07774 (Figure 2A). qRT-PCR showed that the LOC_Os08g07740, encoding a histone-like transcription factor and archaeal histone, had a large expressional difference between 1880 and JH2B (Figure 2B–E). And it was reported a flowering suppressor named EF8/LHD1 [45,46], we deduced that the LOC_Os08g07740 was responsible for OsHd8.
Figure 1. Genetic analysis of heading date in 1880 and JH2B. (A) The phenotype of the 1880 and JH2B for heading date. Scale bars = 5 cm; (B) Statistics on the heading date of 1880 and JH2B; Values are means ± SD (n = 5), *** indicates a significant difference at $p < 0.001$ by t-test; (C) Distribution of heading date in the F2 population derived from 1880 × JH2B.

Figure 2. Identification of candidate heading date gene for OsHd8. (A) Fine mapping of OsHd8. The OsHd8 locus was detected on chromosome 8. Positional cloning narrowed to a 31.8 kb region between marker SNP7730 and SSR-1. The red colors marked gene LOC_Os08g07740 was the candidate gene; (B–E) Comparison of the relative expression levels of four candidate genes among 1880 and JH2B. Values are means ± SEM (n = 3), The asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; Student’s t-test).

3.2. OsHd8 Encodes a Transcriptional Repressor

To investigate whether LOC_Os08g07740 was responsible for the phenotypic changes, a 4.7 kb genomic fragment, including a 2 kb upstream regulatory sequence, a 1.7 kb gene coding region, and a 1 kb downstream fragment of OsHd8 from 1880, was cloned into the vector pCAMBIA1301, and introduced into JH2B, through agrobacterium-mediated transformation. Compared to JH2B, the heading date of transgenic complementary plants was delayed by about one week and showed an increased expression level (Figure 3A–C).
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Figure 3. Effect of OsHd8 on heading date in rice. (A) and (D) Comparison of heading date of JH2B, OsHd8 complemented transgenic plants (Com-1-Com-3) and OsHd8 overexpression transgenic plants (OEHd8-1-OEHd8-3). Scale bars = 5 cm; (B) and (E) Statistical analysis of heading date of JH2B, OsHd8 complemented transgenic plants and OsHd8 overexpression transgenic plants. Values are means ± SD (n = 5), The asterisks indicate significant differences (**, p < 0.01; ***, p < 0.00; Student’s t-test); (C) and (F) Expression levels of OsHd8 in the JH2B, OsHd8 complemented transgenic plants and OsHd8 overexpression transgenic plants. Values are means ± SEM (n = 3), The asterisks indicate significant differences (**, p < 0.01; ***, p < 0.00; Student’s t-test).
To further validate the function of LOC_Os08g07740, an overexpression construct was transformed into JH2B. The OsHd8 transcript level increased by about fivefold, and the heading date was delayed by 15 to 35 days compared with control plants (Figure 3D,E). These results demonstrated that LOC_Os08g07740 is OsHd8, which is essential for regulating the heading date in rice.

To further investigate the function of OsHd8, protein subcellular localization was performed and found that it was located in the nucleus (Figure 4A); spatiotemporal analysis showed that OsHd8 was expressed in all tissues, including the roots, young leaves, culms, and panicle (Figure 4B), corresponding to the A protein-BLAST(BLASTp) at NCBI online revealed that OsHd8 encodes a CBFD_NFYB_HMF domain nuclear transcription factor belonging to the HAP3 subunit [46]. The transcriptional activity assays were then performed in rice protoplasts. The luciferase reporter gene contained five copies of binding sites for GAL4, and the Renilla luciferase gene was used as the internal reference. Compared with the transactivator control constructs GALBD-VP16, GALBD-VP16 fused with OsHd8 induced significantly less LUC activity in rice protoplasts (Figure 4C,D), indicating that OsHd8 functions as a transcriptional repressor.

3.3. Expression Level of OsHd8 Affects Heading Date

To illustrate how OsHd8 regulates the heading date, we compared the genomic sequence of OsHd8 between 1880 and JH2B; no nucleotide difference was detected in the coding regions of OsHd8 (Supplementary Figure S2). Instead, four polymorphisms and one 8-bp InDel were found in the promoters of the two parent lines (Figure 5A).

Figure 4. Molecular characterization of OsHd8. (A) Subcellular localization of OsHd8 (35S: OsHd8-sGFP) in rice protoplasts. Scale bar = 10 μm; (B) OsHd8 expression levels in various organs revealed by qRT-PCR, including roots (R), Flag leaf (FL), stem node (St) and young panicles of different lengths (YP1: 0.5–1 cm young panicle, YP2: 1–2 cm young panicle, YP3: 2–3 cm young panicle, YP4: 3–4 cm young panicle, YP5: 4–5 cm young panicle). Values are means ± SEM (n = 3), ** indicates a significant difference at p < 0.01 by t-test; (C) The main structure of vectors of transcriptional activity assays; (D) The transcription activity in rice protoplasts by co-transformation of different effector vectors with the reporter plasmids and internal control vectors.
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To verify whether the 8-bp InDel or 4 SNPs in the OsHd8 promoter affect the OsHd8 expression, we generated constructs by installing two type promoter fragments into the pGreenII0800-LUC vector and then introduced the constructs into rice protoplasts for transient expression assays. Results showed that 1880 promoter activity was much stronger than that of JH2 (Figure 5B). Furthermore, to validate the promoter activation capacity between 1880 and JH2B, a Dual-LUC assay was carried out in tobacco leaves and found that the 1880 promoter had a greater LUC/REN value than JH2B (Figure 5C), meaning that the sequence variations in the promoter of 1880 led to a high expression of OsHd8, and hence the delayed heading date.

3.4. Comparative Transcriptome Analysis of JH2B and OE-OsHd8

To further explore the regulatory network underlying the OsHd8 function, we performed RNA-sequencing analysis with young leaves from JH2B and OE-Hd8. A total of 1198 differentially expressed genes (DEGs) were identified, of which 825 genes were upregulated and 373 genes were downregulated in the OE-OsHd8 plants. Gene Ontology (GO) assay showed that these DEGs were significantly enriched in terms of the metabolic process, binding and transcription regulator activity (Figure 6A). Further analysis of transcription factors of DEGs revealed that transcription factors associated with flowering were enriched in the OsHd8 pathway, such as the MADS family, HAP2 family, bZIP family and WRKY family (Supplementary Figure S3) [25,47–49]. In particular, several genes controlling the rice heading date, such as GHD7.1, SDG718, OsGI and HDT1 [2,50–52], were differentially expressed in OE-OsHd8 plants just as confirmed with qRT–PCR analysis (Figure 6B). These results demonstrated that OsHd8 could be involved in complicated transcriptional regulation processes governing the rice heading date.
ially expressed in OE-OsHd8 plants just as confirmed with qRT–PCR analysis (Figure 6B). These results demonstrated that OsHd8 could be involved in complicated transcriptional regulation processes governing the rice heading date.

Figure 6. Transcriptome analysis of the DEGs from RNA-sequencing data in heading date between JH2B and OE-OsHd8. (A) Gene ontology (GO) annotation of differentially expressed genes (DEG) in heading date between JH2B and OE-OsHd8. The enriched GO terms of molecular function, biological process and cellular component were listed, and ranged from large to small according to $-\log_{10} p$ value; (B) Comparison of transcriptional expression levels of OsHd8 related genes in heading date between JH2B and OE-OsHd8. Values are means ± SEM (n = 3), The asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ns means no significance; Student’s $t$-test).
3.5. Variations in the OsHd8 Promoter Affect its Expression and Heading Date

In order to understand the effects of the promoter variation on the expression of OsHd8, we then selected 101 rice accessions, including 59 indica (IND), 9 temperate japonica (TEJ), 9 tropical japonica (TRJ), 12 aus (AUS), 2 aromatic (ARO) and 10 admixed (ADM) lines from different countries for clustering analysis (Figure 7A). Results showed that OsHd8 diverges into six haplotypes (Figure 7A), of which the haplotype 2 (Hap2), belonging to JH2B, showed the shortest heading date. 1880 belongs to Hap1, which showed the longest heading date, while Hap3, Hap4 and Hap5 were derived from Hap1 by single-base mutations and had a heading date between the Hap1 and Hap2 (Figure 7B). Further transcriptional analysis of OsHd8 in rice leaves showed that the Hap2 expression was significantly lower than the other five haplotypes (Figure 7C), consistent with the phenotype of the haplotypes. These results indicate that the promoter sequence variation seems significantly correlated with the gene expression and heading date of rice.

Figure 7. Haplotype analysis of OsHd8. (A) Haplotype analysis of OsHd8 from 101 rice varieties; IND means indica, TEJ means temperate japonica, TRJ means tropical japonica, AUS means aus, ARO means aromatic, ADM means admixed; (B) Analysis of heading date in representative varieties with six haplotypes. n = 11 in Hap1, n = 42 in Hap2, n = 27 in Hap3, n = 10 in Hap4, n = 8 in Hap5, and n = 3 in Hap6; (C) The relative expression level of the six haplotypes. Values are means ± SEM (n = 3), The asterisks indicate significant differences (*, p < 0.05; ***, p < 0.001; Student’s t-test).

3.6. OsHd8 Is Subjected to Selection in Cultivated Rice

To investigate the genetic relationship of OsHd8 variations, a total of 3024 cultivated rice from the 3k database (http://www.rmbreeding.cn/index/, accessed on 20 April 2022) and 32 O. rufipogon accessions (http://viewer.shigen.info/oryzagenome/, accessed on 20 April 2022) were selected to analyze the genetic diversity of this gene and its flanking region. The nucleotide diversity value (π) of OsHd8 is lower than its flanking regions in both cultivated and O. rufipogon accessions (Figure 8A, Supplementary Table S3), suggesting that OsHd8 might be subjected to natural selection.
Hd8 (https://snp-seek.irri.org/_snp.zul, accessed on 20 April 2022) and OryzaGenome (http://viewer.shigen.info/oryzagenome/, accessed on 22 April 2022), Genomic sequences of 3024 cultivated and selection analysis of OsHd8. The orange and blue rectangles represent the types (Figure 8C). Meanwhile, 1880 was clustered together with the wild rice, meaning indica categorized into 86 haplotypes, and that the subspecies at the japonica and indica OsHd8 showed that the five parameters in the haplotype and nucleotide FST, Nei’s GST, and Hudson’s GST and HST. Genetic analysis showed that the five parameters in the OsHd8 locus were all greater than 0.25 between indica and japonica subspecies, and the haplotype and nucleotide FST reached to 0.549 and 0.764, respectively, (Figure 8B), suggesting strong genetic differentiation between indica and japonica loci [53]. Genetic analysis with the coding sequence indicated that the OsHd8 locus between indica and japonica subspecies from 3024 cultivated accessions, including the estimates of haplotype and nucleotide FST, Nei’s GST, and Hudson’s GST and HST. Genetic analysis showed that the five parameters in the OsHd8 locus were all greater than 0.25 between indica and japonica subspecies, and the haplotype and nucleotide FST reached to 0.549 and 0.764, respectively, (Figure 8B), suggesting strong genetic differentiation between indica and japonica subspecies at the OsHd8 locus [53].

Phylogenetic analysis with the coding sequence indicated that the OsHd8 could be categorized into 86 haplotypes, and that the indica rice was closer to the wild rice haplotypes (Figure 8C). Meanwhile, 1880 was clustered together with the wild rice, meaning Hd81880 evolved from wild rice O. rufipogon (Figure 8D). These results suggest that Hd81880
originated from wild rice and that at least one mutational event was involved in the origin of \(Hd8^{1880}\) in *indica* rice.

4. Discussion

Flowering is an important trait of plants, and the appropriate flowering time is responsible for the growth and successful sexual reproduction in flowering plants [45]. How to accurately control the flowering time in rice is of great practical significance for the improvement of rice yield. In the present study, we found an elite allele of \(EF8/LHD1/DTH8/Ghd8\) from indica 1880, which delays the heading date by about 27 days, compared to the JH2B (Figure 1) [45,46,54,55]. Previously, the functional \(DTH8\) allele from *cv. Asominori* delays the heading date by about 13 days compared to CSSL61 (1-bp deletion in the exon that carries the nonfunctional \(DTH8\)) [55]. In our study, the newly identified mutations in the promoter reduced the expression level of \(OsHd8\) in JH2B, leading to a shorter heading date (Figure 3). Furthermore, the \(DTH8\) allele from *japonica cv. Asominori* could down-regulate the transcription of \(Ehd1\) and \(Hd3a\) to regulate the heading date, and another \(EF8\) functional allele delays the heading date by regulating the \(Hd3a\) and \(RFT1\). However, our results indicated that \(EF8\) could alter the expression patterns of the other genes, \(GHD7.1\), \(SDG718\), \(OsGI\) and \(HDT1\) (Figure 3). Therefore, we speculate that different alleles of a gene may have different regulatory roles and target genes, resulting in different regulatory mechanisms and phenotypes. Whether differences in gene sequences or genetic backgrounds lead to different allelic effects is an interesting question that deserves further investigation.

The promoter is located in the upstream of the gene coding region and contains many cis-acting elements (CRE), and transcriptional regulation is mainly determined by the promoter CREs [29]. In our study, the promoter activity of 1880 is much higher than that of JH2B (Figure 4B,C) because of the SNP and InDel variations in the JH2B promoter (Figure 4A), of which one SNP at -335 bp (G/A) is located in the ABRE cis-acting element in the JH2B promoter (Figure 7). It has been well characterized that ABRE, as an important response element of abscisic acid (ABA), plays an important role in the regulation of the ABA signal network [56]. When encountered with drought stress, plants may accelerate the initiation of the flowering transformation to shorten their growth cycle through the RCN1 mediated ABA signal process [57]. Interestingly, we found that the ABA response element in the \(OsHd8\) promoter of the *japonica* variety Nipponbare with a short heading date was the same as that of JH2B. Haplotype analysis based on the promoter sequence showed that the JH2B haplotype had the shortest heading date and the lowest expression level (Figure 7). This reminds us that the mutation of the ABA-responsive element of \(OsHd8\) in JH2B may lead to the change in the growth period of rice. However, how ABA response elements regulate the expression of \(OsHd8\) needs to be further validated.

During the evolution of rice, the regional adaptability of cultivated rice is affected by the response to the length of daylight [58]. The heading date is determined by a variety of internal and external signals, including light time, temperature, and hormones [59]. The difference in sensitivity of rice to the photocycle and temperature makes the rice vary greatly in different areas. Many rice lines and wild rice in tropical and subtropical regions, such as *O. rufipogon*, have strong photoperiod sensitivity to flowering. This strong photoperiod sensitivity completely inhibits heading in long-day conditions, allowing it to induce heading only in short daylight conditions [60]. The \(Hd8^{1880}\) allele cloned in this study may be a potential genetic resource. This genotype promotes flowering in long-day conditions and is widely present in the 3K database (Figure 8). With further analysis of the regional distribution map of \(Hd8^{1880}\) and \(Hd8^{JH2B}\) haplotypes, we can see that the \(Hd8^{JH2B}\) haplotype distributes mainly in the higher latitude area (Figure 9), meaning that this haplotype rice may be less sensitive to photoperiod and have expanded more greatly than the other haplotype rice.
Figure 9. Geographical distribution of $Hd8^{1880}$ and $Hd8^{JH2B}$ haplotypes. The orange dots represent the geographical distribution of the haplotype $Hd8^{1880}$, and the cyan dots represent the geographical distribution of the haplotype $Hd8^{JH2B}$.

5. Conclusions

This study identified and mapped a heading date gene, OsHd8, from an early-flowering rice JiaHong2B (JH2B). Four SNPs and one InDel in the promoter region of OsHd8 led to the advance of the JH2B heading date. Comparative transcriptome analysis revealed OsHd8 to be involved in regulation of some floral regulators including GHD7.1, SDG718, OsGI and HDT1. OsHd8 presents strong genetic differentiation between indica and japonica subspecies and shows artificial selection during the domestication of cultivated rice. Our work will provide a valuable heading date gene for rice breeding programs.

Supplementary Materials: The supplementary figures and tables are available online at https://www.mdpi.com/article/10.3390/agronomy12102260/s1. Supplementary Figure S1: ED association analysis of candidate genes in heading date. Supplementary Figure S2: Comparison of CDS sequences of OsHd8 gene in 1880, JH2B and Nipponbare. Supplementary Figure S3: Analysis of transcription factors belonging to differentially expressed genes in JH2B and OE-OsHd8 expression profile by MAPMAN software. Supplementary Table S1: OsHd8 haplotypes in the 101 rice cultivars. Supplementary Table S2: Recombination events between OsHd8 and molecular markers. Supplementary Table S3: The estimated parameters of nucleotide diversity ($\pi$) of OsHd8 and its flanking regions. Supplementary Table S4: Primers used in this study.

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