Ghd8 controls rice photoperiod sensitivity by forming a complex that interacts with Ghd7

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Abstract

Background: Flowering time is one of the most important agronomic characteristics that ultimately determine yield potential and eco-geographical adaptation in crops. Ghd8 and Ghd7, two major flowering genes, have similar functions and large pleiotropic effects in controlling the heading date, plant height and grain yield of rice. However, these gene interactions at the genetic and molecular levels have not been determined to date.

Results: In this study, we investigated the genetic interaction between Ghd8 and Ghd7 by using a set of near-isogenic lines and a panel of natural germplasm accessions in rice. We found that Ghd8 affected multiple agronomic traits in a functional Ghd7-dependent manner. Both functional Ghd8 and Ghd7 are pivotal for rice photoperiod sensitivity controlled by Hd1 and Hd3a. GHD8 could form a heterotrimeric complex with HD1 and OsHAP5b to activate the transcription of Ghd7 by binding directly to the promoter region of Ghd7, which contains the CCAAT-box motif.

Conclusions: The results of this study help to elucidate the genetic and molecular bases of Ghd8 and Ghd7 interactions, indicating that Ghd8 acts upstream of Ghd7 to activate its transcription, which inhibits Hd3a expression and thus affects flowering time and rice adaptation.

Keywords: Photoperiod sensitivity, Flowering time, Molecular interaction, CCAAT-box motif, Rice

Background

Photoperiod sensitivity (PS) is defined as the developmental responses of plants to the relative lengths of light and dark periods and confers on many plant species the ability to adapt to a range of growing season periods by means of adjusting flowering time. Flowering time or heading date (HD) is one of the most important agronomic traits in crops. The probable initiation of flowering time in response to climate change largely determines the crop yield potential [1]. This property is observed because a positive correlation was found between grain yield and flowering time in various natural conditions [2], and either too early or too late flowering might cause reduced grain yield. It has been reported that the photoperiod insensitivity in crops due to loss-of-function mutants or weak alleles of flowering time genes restricts their distributions at specific environmental conditions, such as high latitudes [3–6].

The regulatory mechanisms of flowering time have been extensively studied in Arabidopsis and rice [7, 8]. As a short-day plant, rice (Oryza sativa L.) can flower promptly under short-day (SD) conditions and flower relatively late under long-day (LD) conditions. Two independent gene pathways have been reported to be involved in regulating flowering time under both conditions. The OsGI-Hd1-Hd3a (rice GIGANTEA, Heading date 1 and Heading date 3a) signaling pathway in rice is evolutionarily conserved as the GI-CO-FT (GIGANTEA, CONSTANS, and FLOWERING LOCUS T) pathway in Arabidopsis. Among these genes, the expression of florigen genes in the downstream pathway, such as Hd3a/FT plays central roles in determining flowering time. High expression of Hd3a/FT strongly accelerates flower time, and downregulation of its expression delays or prevents flowering [9, 10]. In recent years, several novel flowering genes have been identified in rice; they have no orthologs in the Arabidopsis genome.
and constitute a rice-specific flowering pathway. For example, Ghd7 (Grain number, plant height and heading date 7), a homolog group of CO, CO-like, and TOC1 (CCT)-domain proteins, was identified as a repressor of flowering through inhibiting Hd3a under LD conditions [11]. Ehd1 (Early heading date 1) was identified as the rice-specific flowering signal integrator and acts upstream of Hd3a [12]. In addition, several flowering time genes have recently been identified to participate in either of the two main independent signaling pathways or even link them. Ghd7.1/OsPRR37 harboring a conserved CCT domain was reported to inhibit Ehd1 and Hd3a only under LD conditions but was independent of Hd1. Ghd8/DTH8 (Grain number, plant height and heading date 8), encoding a CCAAT-box binding factor, known as a HAP3/NF-YB protein, was identified as a major effect locus affecting flowering with the dual function to inhibit flowering under LD conditions and promote flowering under SD conditions by regulating Ehd1 and Hd3a. Moreover, GHD8, HD1 and OsHAP5/NF-YC subunits could form a heterotrimeric complex to bind the CORE element at the promoter of Hd3a to directly regulate its expression [6]. Hd1 is a gene that was reported to genetically interact with other flowering time genes, such as Hd2/PRR37, Ghd8 (DTH8/LHD1/Hd5/LH8), Hd6, SE5 and Ghd7 [13–23]. Some of these interactions were further validated at the molecular level, showing a complex of protein-protein interactions to regulate the expression of downstream genes. For example, HD1 and GHD7 proteins form a complex to specifically bind to a cis-regulatory region in Ehd1 and repress its expression [24, 25]. The revelation of interaction among genes at the genetic and molecular levels has considerably enhanced our understanding of the regulatory networks for flowering time.

Ghd7 and Ghd8 are two major genes identified recently with the same pleiotropic effect on the number of grains, plant height and heading date in rice. Previous results showed that a strong genetic interaction exists between Ghd7 and Ghd8, which seriously inhibits flowering time in the rice natural population [26]. The interaction effect could be strongly enlarged by a functional Hd1. However, it remains unknown whether and how these two genes Ghd7 and Ghd8 interact at the molecular level. To address this question, a set of near-isogenic lines (NIL) and a rice core collection panel were first used to investigate the genetic interaction effect of Ghd7 and Ghd8. Then, transcription analysis, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assays were conducted for a likely molecular interaction. Our results revealed that Ghd8 induces the transcription of Ghd7 via GHD8-OsHAP5b-HD1 complex binding to the specific CCAAT-box region in the Ghd7 promoter. Under both SD and LD conditions, Ghd8 might form a complex with OsHAP5B and HD1 activates the transcription of Ghd7 to inhibit the expression of Ehd1 and Hd3a, leading to late flowering. Both functional Ghd8 and Ghd7 are pivotal for rice PS controlled by Hd1 and Hd3a. These results regarding molecular and genetic interactions provide new insights into the gene-regulatory networks controlling flowering time and adaptation in rice.

Results
Ghd8 acts on agronomic traits depending on functional Ghd7
Both Ghd8 and Ghd7 have been identified as major effect loci, exhibiting pleiotropy in affecting heading date (HD), plant height (PH) and grain yield in rice [11, 27]. The significant interaction also affected heading date [26]. To better understand how this interaction acts on different agronomic traits, we developed an F2 population derived from a cross between near-isogenic lines NIL-Ghd8 and NIL-Ghd7 within the common Zhenshan 97B (ZS97) background. The genetic analyses showed that Ghd8 and Ghd7 interact significantly to affect HD (p = 0.005) and PH (p = 0.026) in the segregating population (Fig. 1a and b). Significant difference (p < 0.001) in the three traits HD, PH and spikelet number per panicle (SNP) was detected among the three genotypes at Ghd8 within the functional Ghd7 background (Fig. 1a–c). However, no significant difference was found in the assayed traits within the nonfunctional ghd7 background.

The significant interaction of Ghd8 and Ghd7 was also revealed in a core collection panel consisting of 196 rice varieties. The Tukey honest test (α = 0.05) showed that the varieties carrying Ghd8ghd7 (only Ghd8 is functional) were not significantly different in HD, PH and SNP compared with the varieties carrying ghd8ghd7 (both alleles are loss of function) under natural LD conditions (Fig. 1d–f). However, the varieties carrying Ghd8Ghd7 showed significantly delayed HD and increased PH and SNP compared with the ghd8ghd7 and Ghd8ghd7 varieties. In particular, the varieties carrying Ghd8Ghd7 exhibited a significant delay in HD and an increase in PH compared with ghd8Ghd7. These results indicate that Ghd8 affected agronomic traits in a functional Ghd7-dependent manner. Hence, the results confirmed that Ghd8 genetically interacts with Ghd7 delaying flowering time and increasing plant height in both homogeneous and heterogeneous backgrounds.

Interaction of Ghd8 and Ghd7 is pivotal for Hd1 and Hd3a to control PS
Photoperiod sensitivity is an important factor of rice cultivars to adapt to variable environments. It has been reported that Hd1 is a key gene that largely determines
Given that both Ghd7 and Ghd8 are involved in the Hd1-Hd3a regulatory pathway [25, 29, 30], we tested whether the interaction between Ghd7 and Ghd8 or Ghd7 and Ghd8 alone can affect the function of Hd1 on PS. The core collection was planted under both LD and SD conditions. The PS index of each variety was calculated by the formula \( \frac{|\text{HD}_{LD} - \text{HD}_{SD}|}{\text{HD}_{LD}} \) (Additional file 1: Table S1). A large variation in PS was observed in the rice core collection. Ghd8Ghd7Hd1 (all are functional alleles) revealed significantly higher PS on average than Ghd8Ghd7hd1 (hd1 is nonfunctional) (Fig. 2a), suggesting that Hd1 is the key gene determining PS variation, as reported before [28]. Haplotype Ghd8Ghd7Hd1 also showed significantly higher PS than haplotype ghd8Ghd7Hd1, and the latter revealed a slightly higher PS that was not significant than haplotypes Ghd8ghd7Hd1 and ghd8ghd7Hd1. These results indicate that functional Ghd8 and Ghd7 and their interaction were required for the function of Hd1 on PS.

In addition, Hd3a, the other gene determining the PS of rice, also showed a similar pattern (Fig. 2b), suggesting that both functional Ghd8 and Ghd7 and their interaction played an important role in affecting the expression level of Hd3a on the control of PS. The varieties carrying functional Ghd8, Ghd7, and Hd3a promoter belonging to the high expression type [31] revealed a
significantly higher level in the average PS than the other haplotypes. However, this significance was not observed in the varieties carrying functional Ghd8 and Ghd7 along with the promoter of Hd3a belonging to the low expression type.

In addition, a segregating population segregated at three loci, Ghd7, Ghd8 and Hd1, within the homogeneous background of ZS97 was used to analyze the effects of interaction among Ghd8, Ghd7 and Hd1. The analysis of variance among genotypes showed that under natural LD conditions, the functional Hd1 (ZS97 allele) showed the strongest effect to delay HD (average = 160 d) in that genotypes carrying the Ghd8Ghd7Hd1 allele combination when both Ghd7 (9311 allele) and Ghd8 (9311 allele) were functional; however, the ghd8Ghd7Hd1 or Ghd8ghd7Hd1 genotypes carrying either single functional Ghd7 or Ghd8 exhibited earlier heading dates (Additional file 3: Figure S1). These results further indicate that the interaction between Ghd8 and Ghd7 dramatically increased the Hd1 effect on the control of heading date in rice.

**Fig. 2** Genetic interaction between Ghd7 and Ghd8 conditioned Hd1 and Hd3a affecting PS in rice. (a) Comparison of photoperiod sensitivity (PS) index among seven genotype types classed by three functional genes (Ghd7, Ghd8, Hd1) in 196 varieties as shown by the violin box. Ghd8ghd7hd1 was not available in the core collection. (b) Comparison of PS among eight genotype types classed by two genes (Ghd7, Ghd8) and the Hd3a promoter in 139 out of the 196 varieties. The promoter types of Hd3a were selected based on the previous report [31]. The up and down arrows indicate the high and low expression levels of the Hd3a types. Fifty-seven varieties with nonfunctional hd1 were not included in the (b) analysis. The red plus symbol indicates the average of PS. For Ghd8, Ghd7 and Hd1 genes, the “+” and “-” symbols at the bottom of the plots indicate the functional and nonfunctional alleles. The different letters on the top of the plots indicate the significant differences in PS among haplotypes by Tukey’s HSD test (α = 0.05). Because there was only one variety, haplotypes ghd8ghd7hd1 and Ghd8ghd7Hd3a (high expression) were not used in the analysis.
Ghd8 upregulates Ghd7 expression to inhibit Hd3a

To explore the molecular basis of the interaction, transcription levels of five flowering-related genes (Ghd8, Ghd7, Hd1, Ehd1 and Hd3a) were determined in 35-day-old seedling leaves of four NILs (Ghd8Ghd7, NIL (Ghd8ghd7), NIL (Ghd8Ghd7), NIL (Ghd8Ghd7)) (Fig. 3a). NIL (ghd7ghd8) flowered at a similar time under both controlled SD (14 h dark: 10 h light) and LD conditions (10 h dark: 14 h light), showing insensitivity (PS index = 0.01) to the photoperiod change (Fig. 3b), although it carries the functional Hd1 alleles. NIL (Ghd8Ghd7) significantly delayed HD compared with the other three NILs (ghd8ghd7, Ghd8ghd7, and ghg8Ghd7) under both SD and LD conditions and displayed the highest PS (PS index = 0.44). In particular, NIL (Ghd8Ghd7) did not flower, even after 180 d under LD conditions (Fig. 3b). Compared with NIL (ghd8ghd7), NIL (gh8Ghd7) showed no significant difference in HD under SD conditions but significantly delayed HD under LD conditions. In contrast, NIL (Ghd8ghd7) delayed HD under LD conditions while promoting HD under SD conditions, which resulted in higher PS than NIL (ghd8Ghd7). Relative expression analyses of the five flowering genes confirmed that the transcription level of Ghd7 was barely detected in NIL (ghd8ghd7) and NIL (Ghd8ghd7) due to the deletion of the Ghd7 gene fragment in ZS97 [11]. However, Ghd7 in NIL (Ghd8Ghd7) was expressed at a significantly higher level than that in NIL (ghd8Ghd7) under both SD and LD conditions (Fig. 3c). In addition, Ehd1 and Hd3a expression levels in NIL (Ghd8Ghd7) showed a serious inhibition compared with that in the other NILs, which was in line with a severe delay in flowering time in NIL (Ghd8Ghd7) (Fig. 3b). The relative expression level of Hd1 showed no significant difference among the four NILs under both SD and LD conditions, indicating that Ghd8 and Ghd7 did not affect the transcription level of Hd1. These results suggest that Ghd8 might play a role in activating the transcription of the floral repressor Ghd7 to suppress the expression of the downstream genes Ehd1 and Hd3a under both LD and SD conditions, leading to late flowering.

GHD8 mediated by HD1 binding to the promoter of Ghd7

To understand how Ghd8 interacts with Ghd7 at the molecular level, we first conducted yeast two-hybrid assays for the protein interaction between GHD8 and GHD7. However, the assays did not reveal a direct interaction between them (Additional file 4: Figure S2a). It has been suggested that either Nuclear Factor A (NF-YA) could interact with NF-YB/NF-YC to form the NF-Y trimeric complex involved in transcriptional activation by binding specific cis-regulatory elements, such as the CCAAT-box or the CORE element [6, 32, 33]. To address whether Ghd8 (NF-YB) regulates the expression of Ghd7 by directly or indirectly binding the specific DNA region at the promoter of Ghd7, we investigated the CCAAT box and CORE element within the 2.5-kb promoter region of Ghd7 by using the online promoter database PLACE [34]. The Ghd7 promoter region contains two CCAAT-box motifs at the proximal and distal regions: −355 bp and −1603 bp upstream of the start codon (ATG) and one CORE1-like motif CATCCACA/TGTGGATG detected at −285 bp [35, 36]. We then developed GHD8-GFP transgenic plants and conducted ChiP assays with extracts from these transgenic lines. The overexpression GHD8-GFP transgenic line showed a significant difference in HD with an approximately 3-d delay compared with the wild type under natural LD conditions (Fig. 4), indicating that the GHD8-GFP protein had biological function. The precipitated products in the presence of the antibody GFP and input (no antibody) were analyzed by qPCR using a set of 9 pairwise primers (Fig. 5a, b) corresponding to different regions in the promoter region. The ChiP assays showed that the adjacent fragments cp1 and cp2 covering the CCAAT-box at −355 bp and CORE1-like motif at −285 bp upstream of ATG displayed the highest enrichment in GHD8-GFP relative to that in the input control among all of the primer sets (Fig. 5b). These results indicate that the GHD8 protein might be involved in binding the CCAAT-box and/or CORE-like motif in the Ghd7 promoter to activate its transcription.

We also found that GHD8 directly interacts with OsHAP5b in both yeast two-hybrid assays and pull-down experiments (Additional file 4: Figure S2b and c). The genetic interaction study revealed that functional Ghd8 and Ghd7 are essential for Hd1 controlling PS in rice (Fig. 2, Additional file 3: Figure S1), implying these three genes may work together to regulate flowering time. To test if the complex formed by GHD8 and OsHAP5b recognizes the CCAAT-box at the promoter of Ghd7 by interacting with HD1, EMSA was performed by using the F1 probe that overlaps with the cp1 and cp2 fragments (Fig. 5a, c). The EMSA results revealed that the recombinant HD1 (MBP-HD1) alone could directly bind to the F1 fragment in vitro with a weak binding ability, and the excess unlabeled F1 fragment (competitor) inhibited the binding (Fig. 5c, d). We found that MBP-HD1 incubated either with His-GHD8 or with His-GHD8 and His-HAP5b could bind to the F1 fragment with different affinities in the EMSA assay (Fig. 5d). Moreover, a considerably stronger binding signal was observed when GHD8, HD1 and OsHAP5b proteins were incubated together with probe F1 (Fig. 5d), which is consistent with a previous study demonstrating that HD1 interacts with GHD8 and OsHAP5b to form a trimeric complex to bind the CORE motif in rice [30]. This result was also confirmed by using the competition assays with the unlabeled F1 fragment and the probe MF1 (a mutated F1 probe lacking the CCAAT element) (Fig. 5e).
Fig. 3 (See legend on next page.)
Discussion

Molecular interaction of Ghd8 and Ghd7

Ghd7 and Ghd8 have been reported as the major genes determining flowering time and plant growth [11]. In the present study, we demonstrate that Ghd8 genetically interacts with Ghd7, delaying heading date in near-isogenic lines and in the rice core collection. Importantly, we found that the transcript level of Ghd7 was activated by Ghd8 through the GHD8-HAP5b-HD1 complex.

Under LD conditions, Ghd7 strongly represses the expression of Ehd1, leading to decreased expression of floral gene Hd3a to inhibit flowering in rice [11]. Recent studies have revealed that Ghd7 is controlled at the transcriptional and posttranslational levels by other flowering time genes, such as OsELF3 and Hd16 [29, 37], which suggest that modification of Ghd7 is a critical role in the decision of flowering time in the long-day repression pathway. A regulatory pathway in a previous report showed that Hd1 upregulated the expression level of Ghd7, leading to downregulation of Ehd1 and Hd3a under LD conditions [24]. Additionally, Nemoto et al. reported that the interaction between HD1 and GHD7 proteins resulted in a synergistic repression of Ehd1 to cause a serious delay of flowering time [25]. The GHD7-HD1 complex represses Ehd1 by binding to a cis-regulatory region of Ehd1 [6], which suggested that Hd1 was integrated into the LD repression pathway. Due to the high expression of Ghd7, Ehd1 and Hd3a were repressed to delay flowering [27, 30]. Hence, Ghd7 not only works as a signal integrator to receive the output from circadian clock [37], but also plays a role in transmitting regulation signals of other flowering-related genes, such as Hd1 to Ehd1, to affect flowering time. It is notable that no difference in the Hd3a expression level was revealed in the NILs ghd8Ghd7 and ghd8ghd7, intriguingly, Hd3a was repressed in NIL (Ghd8Ghd7) by approximately eight-fold compared with that in NIL (Ghd8ghd7) under SD conditions (Fig. 3c). These results suggest that Ghd7 did play a role as a repressor of the expression of Hd3a in a dependent manner of functional Ghd8 (Fig. 6). Furthermore, our data revealed the enhanced effect of Ghd8 and Ghd7 interaction on flowering time in the genetic background of ZS97 with a functional Hd1; however, the transcription level of Hd1 remains unchanged in the NILs with the ZS97 background. This finding suggests that Ghd8 might integrate the Hd1-Ghd7 pathway in the regulation of rice flowering through a posttranscriptional mechanism that the protein GHD8 or GHD7 might interact with HD1.

Previously, Ghd8 was shown to play a similar role as Hd1 with repression or activation in flowering time under LD or SD conditions [27, 30]. Hd1 repressed Hd3a under LD conditions or activated gene expression under SD conditions in the presence of the nonfunctional ghd7 [25]. In the current study, the dual function for Ghd8 on flowering was also found to be dependent on the presence of the nonfunctional ghd7 (Fig. 3b, c). In the comparison between NIL (ghd8ghd7) and NIL...
(Ghd8ghd7) with the functional Hd1 background, Ghd8 caused early flowering under SD conditions. Consistently, the Hd3a expression was much more activated in NIL (Ghd8ghd7) than that in NIL (ghd8ghd7). Previous study also revealed that under SD conditions, Hd1 could activate the expression of Ehd1 and Hd3a regardless of Ghd7 functions [11]. In addition, it was reported that HD1-containing NF-Y complex has the capacity to bind a conserved response element in the Hd3a promoter [6]. These results suggest that Ghd8 acts as an activator and might work together with Hd1 to regulate Hd3a under SD conditions (Fig. 3). As mentioned above, the GHD7-HD1 complex could bind the Ehd1 promoter under LD conditions [25]. The GHD8-HD1 complex promoted the flowering time through regulating Hd3a expression [30]. And GHD8-HAP5b-HD1 complex may activate Ghd7 expression to enhance its repression effect on flowering time. These data point to the flowering regulatory pathway linking Ghd8 to Hd1 and Ghd7. Taken together, these results indicate that Hd1 not only plays an important role in activating transcription under SD conditions but also incorporates GHD7 protein, inhibiting the expression of Ehd1 in the LD repression pathway. Moreover, Ghd8 could enhance the inhibitory role of Ghd7 in flowering time under both SD and LD conditions (Fig. 3c). These findings suggest that the Hd1...
function become flexible to affect flowering time in terms of the photoperiod change through interacting with other transcription factors like Ghd8 and Ghd7.

**GHD8-OsHAP5b-HD1 binds the CCAAT-box**

Ghd8 encodes a homolog of HAP3 or NF-YB subunit, together with HAP2 and HAP5 or NF-YA and NF-YC, constitutes the trimeric complex to bind the CCAAT-box at the promoter to activate the gene transcription [33]. However, in the absence of NF-YA, other transcription factors, such as CO or bZIP, also interact with NF-YB and NF-YC to form new complexes, leading to different binding sites that do not strictly bind to the CCAAT-box [30, 38–41]. For example, the CCT domain of CO and CO-like protein could interact with the complex of NF-YB and NF-YC to bind the CORE-element in *Arabidopsis* [35, 42]. In rice, the GHD8-OsHAP5b-HD1 complex has been reported to directly bind the CORE1 element on the promoter of *Hd3a* to regulate flowering [30]. In the current study, GHD8-OsHAP5b was found to interact with HD1 to form a trimeric complex to bind the CCAAT-box at the −355 bp site of the Ghd7 promoter. HD1 alone or the HD1-Ghd8 dimer were able to directly bind to the DNA fragment F1 that harbors the CCAAT-box, but this fragment did not cover the CORE1-like motif sequences (Fig. 5). Therefore, we propose that GHD8-HD1 or HD1 protein can recognize the CCAAT motif at the −355 bp site.

In addition, the trimeric complex of NF-YB-NF-YC-CO appears more important to the binding of the CORE motif in *Arabidopsis* [35], and even mutation of any one of them can completely eliminate the binding ability of the complex. In contrast, the NF-YB1 subunit alone was reported to directly bind downstream genes without the assistance of other NF-Ys in the endosperm in rice [40, 43]. Our study revealed the low binding affinity of HD1 and HD1-GHD8 dimer on the CCAAT-box motif, suggesting that HD1 protein might be able to bind both the CCAAT-box motif and the CORE1 motif and have an overlapping function with OsHAP2/NF-YA and compete with these subunits to recognize the CCAAT-box motif [44]. Although the role of the CORE1-like motif in the binding stability of the GHD8-OsHAP5b-HD1 complex needs to be further investigated, the molecular interaction between Ghd8 and Ghd7 to control of *Hd3a* expression supports that the GHD8-OsHAP5b-HD1 regulatory complex is involved in the Ghd7 repression pathway. These results suggest the existence of multiple targets for the GHD8-OsHAP5b-HD1 complex that fine tune the spatio-temporal regulation of gene expression in the flowering network, which might have a greatly contributed to the adaption of rice to various cultivation areas.

**Implications of Ghd7 and Ghd8 interaction**

Asian cultivated rice has two subspecies: *indica* and *japonica*, both of which are distributed in different eco-geographic environments [31]. In the present study, Ghd8 and Ghd7, as well as their interaction effect, were detected in response to the specific photoperiod conditions. The varieties with both weak functional Ghd7 and Ghd8 alleles dominate in the northern region between 35°N and 45°N, where farmers usually practice single
planting in the summer cultivation season of long day-length (Additional file 5: Figure S3). The majority of varieties planted there are *japonica* subspecies [11, 27] and show a relatively low PS (average PS index = 0.2) (Additional file 1: Table S1). In parallel, the varieties with strong functional *Ghd7* and *Ghd8* with a relatively high PS are mainly found in subtropical and tropical regions between 25°N and 15°N. Additionally, a portion of varieties that contain both nonfunctional alleles *ghd7* and *ghd8* occur predominantly along 25°N latitudes (Additional file 5: Figure S3). This finding suggests that nonfunctional *ghd7* and *ghd8* are beneficial for the rapid growth of varieties required in this zone, where multiple plantings occur.

Notably, almost all of the northern *japonica* varieties carrying both functional alleles of *Ghd7* and *Ghd8* have functional *Hd1* (Additional file 5: Figure S3). However, these varieties flowered early (68–85 d) in natural LD conditions. This unexpected observation is different from the late heading in the NILs with the *indica* background, although they carry all functional alleles of *Ghd7*, *Ghd8* and *Hd1*. One explanation is that the *japonica* varieties might have divergent *Ghd7*, *Ghd8* or *Hd1* alleles with a reduced/weaker function that probably facilitates *japonica* rice adaptation to the northern/high latitudes [11, 27, 31, 45, 46]. Our study indicates that a high disequilibrium linkage of *Ghd7* and *Ghd8* exists in *japonica* rice (*p* < 0.01), and *Ghd8* or *Ghd7* also occurs in linkage disequilibrium with *Hd1* and/or *Hd3a* in either *indica* or *japonica*. This high linkage relationship among these genes suggests that some preferable genes in the regulation network might be selected together for suitable flowering (Additional file 6: Figure S4). The other possible explanation is that some flowering gene interaction unidentified in rice helps to counteract severely delayed flowering that arose by functional *Ghd7*, *Ghd8* and *Hd1* [23, 29].

There is a significant association between flowering time and grain yield in rice. A longer flowering time is usually associated with higher grain yield. However, there is a trade-off between increased yield potential and delays in HD. Therefore, in terms of the action of *Ghd7* and *Ghd8*, which play a crucial role in the regulation of flowering, these genes should be considered together in high-yield breeding programs, especially for those varieties used in the low latitude regions, where multiple plantings are commonly applied for obtaining high yield per area. As rice is the main source of daily calories, deciphering the combined effects of *Ghd8*, *Ghd7*, and *Hd1* in flowering regulatory pathways will help elucidate the adaptation mechanism to various photoperiod conditions and will be beneficial in molecular design for the development of rice varieties with high yield potential.

**Conclusions**

This study investigates the genetic interaction effect between two major genes, *Ghd8* and *Ghd7*, by using the segregating population generated from the cross between NIL lines and a natural germplasm panel in rice. The molecular basis of their interaction was further identified by using a series of experiments, including EMSA and ChIP. We found that *Ghd8* regulates transcript of *Ghd7* through the GHD8-OsHAP5b-HD1 regulatory complex. Thus *Ghd8* and *Ghd7* together with *Hd1* largely explain seriously delayed flowering time, increased plant height, and improved yield potential in rice varieties. We also found that both functional *Ghd8* and *Ghd7* are pivotal for rice PS controlled by *Hd1* and *Hd3a*, which is highly important for rice varieties to adapt to different environments. These findings provide insights into the molecular and genetic bases of the gene interaction on crop productivity and adaptation and reveal that the selectivity and combination of different functional alleles could be highly useful for improving yield potential in rice breeding programs.

**Methods**

**Plant material and growth conditions**

The panel of 196 rice accessions was used for nucleotide sequencing analysis [47, 48]. These accessions were obtained from China National Rice Research Institute and the International Rice Research Institutes. The varieties were grown in Wuhan (N30.52, E114.3) under natural LD conditions and in Hainan (N18.48, E110.02) under natural SD conditions for the measurements.

Two advanced backcross lines harboring the genomic regions with either functional *Ghd7*, *Ghd8* or *Hd1* were used to generate F1 hybrids [20]. The F1 plants were then backcrossed with ZS97 twice to create the BC2F1 generation. The segregating population of BC2F2 containing approximately 200 individuals was grown in Hainan. The SSR markers RM5436, PID2, and RM121 were used to select the heterozygous segments at *Ghd7*, *Ghd8* and *Hd1* during the crossing scheme. The four homozygous genotypes of *Ghd8Ghd7*, *Ghd8ghd7*, *ghd8Ghd7* and *ghd8ghd7* with the common background of ZS97 were selected as the near-isogenic lines (NILs) in which *Hd1* is functional.

To compare the HD and PH of the NILs under different light/dark conditions, 10 plants for each NIL were grown in LD (14 h light: 10 h dark) and SD (10 h light: 14 h dark) conditions in growth chambers. Flowering time was scored from 8 individuals per line. HD was defined as the time when the first panicle appeared from the flag leaf.

**DNA sequencing**

The rice core collection was genotyped by sequencing at the coding region of *Ghd7*, *Hd1*, *Ehd1*, *Hd3a* and *Ghd8*
covering the functional site described in recent papers [11, 12, 27]. The PCR amplified fragments were sequenced directly using BigDye Terminator Cycle Sequencing v3.1 (Applied Biosystems, Foster, USA) after digestion and purification according to the manufacturer’s specifications. The primers used are listed in Additional file 2: Table S2.

Gene expression analyses
Leaves from the main culm of NILs of 35-day-old plants were harvested to analyze the transcription levels of Ghd8, Ghd7, Hd3a, Ehd1 and Hd1. The NILs were grown in the growth chambers respectively under SD and LD conditions at 30°C in the light and 26°C in the dark. Samples were collected at Zeitgeber time (ZT) 4 under SD conditions and at ZT 8 under LD for gene expression analysis. ZT 0 indicates dawn. The samples were collected for RNA extraction using an RNA extraction kit (TRIzol Reagent, Invitrogen). The time points for sampling corresponded to the expression peak for each gene according to the reports [11, 27]. Approximately 2 μl of RNA was used to synthesize the cDNA in a 20 μl reaction volume by using SuperScriptII reverse transcriptase (Invitrogen) after treatment with DNase I to remove the contaminating genomic DNA. Real-time PCR (RT-PCR) was performed in a total volume of 25 μl on an Applied Biosystems 7500 Real-Time PCR System, which contained 2 μl cDNA, 2 μl primers (0.4 μM), 8.5 μl distilled water and 12.5 μl of SYBR® Green PCR Master Mix (Applied Biosystems) for each reaction. The expression data were normalized by using the ubiquitin gene with the relative quantification method [49]. All experiments were conducted in at least three biological and three technical replicates. The primers used for the transcription analyses are listed in Additional file 2: Table S2.

Yeast two-hybrid assay
The protein-coding regions of Ghd7, Ghd8 and OsHAP5b were amplified using gene-specific primers with added restriction enzyme sites, respectively (Additional file 2: Table S2). Then, the Ghd7 or OsHAP5b product amplified by PCR was fused into the activation domain (AD) vector pGADT7 as a prey system with EcoRI and Xhol sites, and the Ghd8 product with EcoRI and BamHI sites was fused to the DNA-binding domain (BD) vector pGBKTK7 as bait system. All constructs were verified by sequencing. The co-transformation of two plasmids carrying the Ghd7, OsHAP5b and Ghd8 genes into the AH109 yeast strain and cell culture were performed according to the manufacturer’s protocols (Matchmaker Gold Yeast Two-Hybrid System, Clontech). Constructs of pGBKTK7–53 (pBD-53) and pGADT7-T (pAD-T) served as positive controls, and constructs of pGBKTK7-Lam and pGADT7-T (pAD-T) served as negative controls.

In vitro pull-down assay
The coding region of Ghd8 was cloned into the pET-32a vector (Novagen) and pGEX-6P-1 vector (GE Healthcare) with EcoRI and Xhol sites, respectively. To obtain the OsHAP5b protein, we used the same method for GHD8. The recombinant expression vector was expressed in Escherichia coli Transetta (DE3) cells (Transgen). The pull-down experiment was performed as described previously [50]. In brief, supernatants with equal amounts of Glutathione S-transferase (GST) or OsHAP5b-GST with GHD8-His recombinant proteins were incubated for 6 h at 4°C in a total volume of 2 ml of pull-down buffer (20 mmol Tris-HCl, pH 8.0, 200 mmol NaCl, 1 mmol EDTA, 0.5% Lgepal CA-630 and protease inhibitor), after which 200 μl of GST resin was added (GE Healthcare; 17–5132-01) and the mixture incubated for 2 h at 4°C. The binding reaction was then washed 5 times (10 min each time at 4°C) using the pull-down buffer. After extensive washing, the pulled-down proteins were eluted by boiling at 95°C for 10 min, separated on 12% SDS-PAGE and detected by immunoblots using an anti-GST antibody (Abcam; ab19256) and anti-His antibody (Abcam; ab9108), respectively.

ChIP (chromatin immunoprecipitation)
For ChIP assays, wild-type and GHD8-GFP transgenic lines were used for chromatin extraction and immunoprecipitation following the method described [51]. Briefly, young leaves from approximately 35-day-old seedlings were collected under LD conditions (15 h light: 9 h dark). After treated with formaldehyde to cross-link the proteins to the DNA, the cross-linked lysate was sonicated to shear DNA to the smaller fragment size. The soluble chromatin fragments were isolated and purified for the following steps. Immunoprecipitation with anti-GFP (Abcam; ab290) was performed for wild-type and GHD8-GFP transgenic lines with 3 repeats. The precipitated DNA was analyzed by quantitative RT-PCR using specific primer sets listed in Additional file 2: Table S2, designed to cover the CCAAT-box element within a 2-kb primer region upstream of ATG of Ghd7.

To construct the GHD8-GFP fusion, maize (Zea mays) ubiquitin promoter-GFP cassette from pU1301 was used to substitute the GUS fragment of pCAMBIA1391Xb to create a modified pCAMBIA1391Xb [51]. The conservative domain within the Ghd8 coding region involving in the binding and interaction function was amplified and inserted into the KpnI and BamHI sites of the modified pCAMBIA1391Xb to obtain the construct Ubi::GHD8-GFP for rice transformation. The Agrobacterium mediated genetic transformation was used to generate the
transgenic plants with Ubi::GHD8-GFP construct in the Nipponbare background.

**Electrophoretic mobility shift assay (EMSA)**
The pET32-based expression vectors for GHD8 and OsHAP5b were used to express recombinant proteins fused to a thioredoxin and a His (polyhistidine)-tag in tandem at their N-terminal ends. An *E. coli* BL21 strain (Transgen) was transformed with vectors and grown at 37 °C. pMAL vector (New England Biolabs, E8000S) was used to obtain the recombinant HD1 proteins with the maltose-binding protein (MBP) at the down-stream. The recombinant proteins pET32-GHD8, pET32-OsHAP5b and pMAL-c2X-HD1 were purified with Ni-NTA agarose (QIAGEN; No. 30210) and amylase resin beads (New England Biolabs) respectively. The *Ghd7* promoter fragment F1 (including the putative binding site CCAAT-box) were produced by annealing of 3′-biotin-labeled oligonucleotides Ghd7F/R (Sangon Biotech), respectively. DNA binding reactions were performed in the presence or absence of unlabeled F1 and labeled mutatated F1 (MF1 and MF2) fragments at room temperature for 20 min in 5 mM Tris, pH 7.5, 25 mM KCl, 0.5 mM DTT, 5 mM MgCl₂, 2.5% glycerol, 0.05% NP-40 and 50 ng/μL poly (dI-dC). We followed the protocol from the LightShift Chemiluminescent EMSA Kit (Thermo; No.20148), and the samples were run on 5% polyacrylamide gels. The primers used for EMSA are listed in Additional file 2: Table S2.

**Linkage disequilibrium analysis**
Linkage disequilibrium analysis of *Ghd7*, *Ghd8*, *OsHAP5b*, *Hd1* and *Hd3a* was performed using the software TASSEL 3.0.147 (http://www.maizegenetics.net/). The sequences including single nucleotide polymorphisms in the promoter region (1.5 kb upstream of ATG), coding region and 3′UTR (1 kb downstream of the stop codon) of these genes in 532 varieties were obtained from the website (http://ricevarmap.ncpgr.cn/django/snp_id/). All SNPs in each gene from the varieties were input into TASSEL to conduct linkage disequilibrium analysis. Out of 532 varieties, 2 groups, each including 295 indica and 156 japonica were also separated for linkage disequilibrium analysis, and a threshold of 1% was set to filter the SNPs with low frequencies.

**Statistical analysis**
Analysis of variance (ANOVA) of gene interaction on the assayed traits in the F₂ segregating population derived from the cross of NIL (*Ghd7*) × NIL (*Ghd8*) was conducted using Statistica software [52]. Tukey’s HSD (honest significant difference) test in JMP software was employed to determine which groups were significantly different [53].
Competition of interests
The authors declare that they have no competing interests.

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