

# Strong photoperiod sensitivity is controlled by cooperation and competition among Hd1, Ghd7 and DTH8 in rice heading

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## Summary

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Received: 16 June 2020

Accepted: 5 September 2020

New Phytologist (2021) 229: 1635–1649

doi: 10.1111/nph.16946

**Key words:** flowering time, heading date, photoperiod sensitivity, rice, short-day plant.

- Rice (*Oryza sativa*) is a short-day (SD) plant originally having strong photoperiod sensitivity (PS), with SDs promoting and long days (LDs) suppressing flowering. Although the evolution of PS in rice has been extensively studied, there are few studies that combine the genetic effects and underlying mechanism of different PS gene combinations with variations in PS.
- We created a set of isogenic lines among the core PS-flowering genes *Hd1*, *Ghd7* and *DTH8* using CRISPR mutagenesis, to systematically dissect their genetic relationships under different day-lengths. We investigated their monogenic, digenic, and trigenic effects on target gene regulation and PS variation.
- We found that *Hd1* and *Ghd7* have the primary functions for promoting and repressing flowering, respectively, regardless of day-length. However, under LD conditions, *Hd1* promotes *Ghd7* expression and is recruited by *Ghd7* and/or *DTH8* to form repressive complexes that collaboratively suppress the *Ehd1-Hd3a/RFT1* pathway to block heading, but under SD conditions *Hd1* competes with the complexes to promote *Hd3a/RFT1* expression, playing a tradeoff relationship with PS flowering. Natural allelic variations of *Hd1*, *Ghd7* and *DTH8* in rice populations have resulted in various PS performances.
- Our findings reveal that rice PS flowering is controlled by crosstalk of two modules – *Hd1-Hd3a/RFT1* in SD conditions and (*Hd1/Ghd7/DTH8*)–*Ehd1-Hd3a/RFT1* in LD conditions – and the divergences of these genes provide the basis for rice adaptation to broad regions.

## Introduction

Photoperiod is an important environmental factor regulating plant growth and development. Many plant species sense day-length to determine when to transition from vegetative growth to reproductive development. Plants are classified as long-day (LD), short-day (SD) and day-neutral species according to how they sense photoperiod to induce flowering (Thomas & Vince-Prue, 1997). Rice (*Oryza sativa*), a SD species, was domesticated from the wild rice species *Oryza rufipogon* in tropical and subtropical Asian regions, and has differentiated into two subspecies, *indica* and *japonica* (Huang *et al.*, 2012). Many landraces in tropical and subtropical regions, such as *O. rufipogon*, have very strong photoperiod sensitivity (PS) for flowering (heading). This strong PS completely inhibits heading under LD conditions (i.e. in the early season) and induces heading only under SD conditions (i.e. in the late season in the tropical and subtropical regions). This gives the plants ample time for vegetative growth before flowering, a successful strategy to fully utilize light resource. However,

the long-term natural and artificial selection that occurred during rice domestication and breeding has resulted in the expanded rice cultivation from its primitive domesticated regions to wider regions (from 53°N to 40°S), and adaptation to different cropping systems (e.g., single- and double-cropping systems, with a single rice planting per year in the mid-season, and two rice plantings in the early and late seasons, respectively), which requires rice cultivars to have various degrees of PS that allows them to flower under different day-lengths (Brambilla & Fornara, 2013; Brambilla *et al.*, 2017).

In rice, a number of heading date genes have been cloned and functionally characterized, showing that rice possesses complicated flowering pathways that are both conservative and unique to those characterized in dicots (Song *et al.*, 2015; Brambilla *et al.*, 2017). An important regulator of photoperiod-sensitive heading is *Heading date 1* (*Hd1*), a homolog of *Arabidopsis thaliana* *CONSTANS* (*CO*) (Putterill *et al.*, 1995; Yano *et al.*, 2000). The tandem-duplicated genes *Heading date 3* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) are orthologs of the *Arabidopsis* florigen gene *FLOWERING*

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*LOCUS (FT)*, and like FT, they function to induce flowering (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007). In contrast to *CO*, which promotes flowering under LD conditions, *Hd1* generally promotes heading under SD conditions but delays heading under LD by up- and downregulating *Hd3a/RFT1* expression, respectively (Yano *et al.*, 2000; Ishikawa *et al.*, 2011; Nemoto *et al.*, 2016).

Some aspects of the flowering pathways in rice differ from those discovered in *Arabidopsis*. For example, the rice heading date gene *Early heading-date 1 (Ehd1)* encodes a B-type response regulator promoting the expression of *Hd3a* and *RFT1* (Doi *et al.*, 2004; Zhao *et al.*, 2015). In addition, several LD-specific repressors of *Ehd1* have been identified, such as *Grain number, plant height and heading-date7 (Ghd7)*; also named *Heading date 4 (Hd4)*, *Days-to-heading on chromosome 8 (DTH8)*; also named *Ghd8/Hd5* and *Pseudo-response regulator 37 (PRR37)*; also named *Hd2/ Ghd7.1* (Xue *et al.*, 2008; Wei *et al.*, 2010; Yan *et al.*, 2011; Koo *et al.*, 2013; Yan *et al.*, 2013; Gao *et al.*, 2014). *Hd1*, *Ghd7* and *OsPRR37* encode CO, CO-like and TOC1 (CCT)-domain proteins, respectively (Yano *et al.*, 2000; Xue *et al.*, 2008; Koo *et al.*, 2013), and *DTH8* is a CCAAT-box-binding transcription factor, also named *OsNF-YB11* (Wei *et al.*, 2010; Yan *et al.*, 2011). These genes are central components in the LD-suppression pathways that influence rice heading date and grain yield, and *Ehd1* seems to function as a signal integrator for multiple regulatory pathways under LD conditions (Tsuji *et al.*, 2011; Shrestha *et al.*, 2014).

Research has been carried out to investigate the mutual regulation of *Hd1*, *Ghd7*, and *DTH8* for flowering (Zhang *et al.*, 2015; Li *et al.*, 2015; Nemoto *et al.*, 2016; Du *et al.*, 2017; Z. Y. Zhang *et al.*, 2017, 2019; B. Zhang *et al.*, 2019). The *Hd1* floral repressor activity basically requires *Ghd7* in LD conditions in functional *DTH8* background (Nemoto *et al.*, 2016). The heading repression in LD by *Hd1* also depends on *DTH8* in functional *Ghd7* background (Du *et al.*, 2017). *DTH8/Ghd8*-repressed heading depends on *Ghd7* in functional *Hd1* background (Wang *et al.*, 2019). But this raises the question, are interactions of any two of the three genes, *Hd1*, *Ghd7*, and *DTH8*, especially in strong PS cultivars, the same when the third gene is nonfunctional? So, the comprehensive mechanism by which these genes interactively control the various extents of PS in the same genetic background, and how the dual activity of *Hd1* is achieved, need to be further addressed. In this study, we identified *Hd1*, *Ghd7* and *DTH8* as major genetic factors controlling strong rice PS. We created a set of eight isogenic lines with all the combinations of the three genes, and investigated the heading date and expression of the heading-related genes of these lines in different day-length conditions. Our results revealed the basic functions of *Hd1*, *Ghd7* and *DTH8* and their collaborative roles. We demonstrate that the evolution of PS in rice populations is mainly attributed to natural allelic variations and genetic interactions of *Hd1*, *Ghd7* and *DTH8*, which is critical for adaptation of rice to wider cultivation areas in different geographic regions.

## Materials and Methods

### Plant materials and growth conditions

Rice (*Oryza sativa* L.) materials used in this study included: the *NHLD* plants (with *Hd1*, *Ghd7* and *DTH8*) and *nhld* plants (with *hd1*, *Ghd7* and *DTH8*), which were selected from an F<sub>5</sub> family from a cross between an *indica* landrace (accession no. I7) and the *indica* cv Qinghuazhan; *Hd1*-transgenic lines with *Hd1*-I7 from I7 and *Hd1*-Nip from a *japonica* cv Nipponbare in the *nhld* background; *Hd1*-, *Ghd7*- and *DTH8*-knockout lines in the *NHLD* background and their crossing progenies; the wild rice and cultivated rice landraces. The rice plants examined under field conditions were grown at the Experimental Station of SCAU, Guangzhou (23.13°N, 113.27°E), China, in the normal rice-growing early season with natural LD (NLD) conditions (13.5–14.0 h day-lengths from mid-May to mid-July as photoperiod-sensing stage), in the late season with natural SD (NSD) conditions (13.5–12.0 h day-lengths from mid-July to October), in artificial LD conditions (ALD, growing from mid-March with supplemental lighting from July to mid-November before dusk to reach a total daylight time of 14.0–14.5 h), and in artificial SD conditions (ASD, with 11.0–11.5 h day-lengths by shading treatment in the late season). In all, 200 plants of the F<sub>5</sub> family and 1049 F<sub>6</sub> plants were used for primary and fine mapping.

Genomic fragments of 4.3 kb of *Hd1* from I7 and Nipponbare, which harbored the coding region, 1.4 kb of upstream sequence, and 1 kb of downstream sequence, were amplified and cloned into the vector pCAMBIA-1300. The constructs were transferred into *nhld* plants by *Agrobacterium tumefaciens*-mediated transformation. For knockout of *Hd1*, *Ghd7* and *DTH8*, the target sites were designed using clustered regularly interspaced short palindromic repeats (CRISPR)-GE/targetDesign (<http://skl.scau.edu.cn/>) (Xie *et al.*, 2017) and genome-targeting constructs were prepared using the pYLCRISPR/Cas9Pubi-H vector (Ma *et al.*, 2015). The constructs were transferred into *NHLD* plants. The target sites were sequenced and analyzed using CRISPR-GE/DSDecodeM (Liu *et al.*, 2015; Xie *et al.*, 2017).

### Trait measurement and data analysis

Heading dates were scored as time (in d) from seed-soaking to emergence of the first panicles. The net genetic effects ( $e$ ) of monogenic *Hd1*, *Ghd7*, *DTH8* in ALD, NSD and ASD were estimated by  $e_{(H)} = D_{(Hgd-7)} - D_{(hgd-8)}$ ,  $e_{(G)} = D_{(bGd-5)} - D_{(bgd-8)}$ , and  $e_{(D)} = D_{(hgD-6)} - D_{(bgd-8)}$ , respectively, where D indicates the average heading date. Their significances at  $\alpha$  probability level were tested by the least significant difference (LSD) method (Yang *et al.*, 2018) with the statistics  $LSD_{\alpha} = t_{\alpha} \sqrt{\frac{2Se^2}{n}}$  (where  $Se^2$  is the variance from experimental error,  $n$  is the average of sample size, and  $t_{\alpha}$  is the critical  $t$ -value under  $\alpha$  probability level and error freedom degree). To evaluate the high-order digenic and trigenic genetic interactions between and among the genes, their  $e$ -values were estimated as  $e_{(HGd)} = D_{(HGd-4)} - D_{(bgd-8)} - e_{(H)} - e_{(G)}$ ,  $e_{(Hgd)} = D_{(Hgd-4)} - D_{(bgd-8)} - e_{(H)} - e_{(D)}$ ,  $e_{(bGD)} = D_{(bGD-2)} - D_{(bgd-8)} - e_{(G)} - e_{(D)}$ , and  $e_{(HGD)} = D_{(HGD-1)} - D_{(bgd-8)} - e_{(H)} - e_{(G)} - e_{(D)} - e_{(HGd)}$

$e^{(HgdD)-e^{(bGD)}}$ , which were tested by  $LSD_{\alpha} = t_{\alpha} \sqrt{\frac{4Se^2}{n}}$  or  $LSD_{\alpha} = t_{\alpha} \sqrt{\frac{8Se^2}{n}}$  (Yang *et al.*, 2018). Correlation between flowering time and gene expression level was examined by Pearson's correlation coefficient.

### Subcellular localization and bimolecular fluorescence complementation (BiFC)

For subcellular localization assay, the *Hd1* coding sequence was cloned into the pLYd1 *GFP* vector carrying the *GFP* tag. The constructs for Hd1-GFP fusion and the nucleus marker TDR:RFP (transiently-expressed TDR:RFP fusion protein) were transiently coexpressed in rice leaf protoplasts by polyethylene glycol treatment (Ji *et al.*, 2013).

For BiFC assay, the *Ghd7* and *DTH8* coding sequences were cloned into the pVN and pVC vectors fusing with the N-terminal or C-terminal sequence of *YFP* to generate *Ghd7-YFP<sup>n</sup>* and *DTH8-YFP<sup>c</sup>*, respectively (Chen *et al.*, 2006). The plasmids were transfected into rice protoplasts followed by an incubation in darkness at 30°C for 15 h, and then their fluorescence was imaged with an Olympus BX61 epifluorescence microscope.

### Transcriptional activation activity assay

This assay was performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA, USA). Plasmids containing the GAL4 DNA-binding domain fused with *Hd1*-truncated fragments were individually cotransformed with pGADT7 into yeast strain AH109. Subsequently, the cells were grown on -2 Synthetic Dropout medium lacking -Leu-Trp, and Ade and -4 Synthetic Dropout medium lacking -Leu-Trp-His-Ade.

### RNA extraction and analysis of gene expression

Fifty-eight-day-old plants grown in ASD, NSD and ALD conditions were used for expression analysis of flowering-time genes. Leaves were harvested every 4 h within 1 d with three biological replicates. Total RNAs were extracted using Trizol (Invitrogen). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays were carried out as described previously (Zhao *et al.*, 2015), using *Actin1* as the internal control.

### In vitro protein pulldown

The coding sequences of *Hd1*, *Ghd7* and *DTH8* were cloned into the pET-32-a, pGET-4T-2 and pMAL-p5X vectors to express the His-Hd1/maltose-binding protein (MBP)-Hd1, glutathione S-transferase (GST)-Ghd7 and MBP-DTH8 proteins, respectively, in *Escherichia coli* Rosetta. The cells were cultured to  $OD_{600nm} = 0.8-1$ , then induced with 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside at 23°C for 6 h. Total proteins were subsequently in a phosphate-buffered solution (2 mM  $KH_2PO_4$ , 8 mM  $Na_2HPO_4$ , and 200 mM NaCl, pH 8.0) by sonication. Total protein concentration was determined by the Bio-Rad protein assay (Bio-Rad).

For *in vitro* pulldown assay, GST-Ghd7 and MBP-DTH8 as baits were immobilized to glutathione agarose beads at 4°C for 2 h. The resins were washed five times with the binding buffer at 300 g 4°C for 2 min. Then MBP-Hd1, His-Hd1 and MBP-DTH8 as prey proteins were added and incubated with bait proteins at 4°C for 2 h. After washing and denaturing, these proteins were pulled down and loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed and detected by Western blot with anti-MBP (1 : 5000, cat. no. HT701; TransGen Biotech, Beijing, China or cat. no. M1321; Sigma), anti-GST (1 : 5000, cat. no. HT601; TransGen Biotech, Beijing, China), and anti-His (1 : 5000, cat. no. HT501; TransGen Biotech).

### Yeast two-hybrid (Y2H)

The *Hd1* coding sequence and its N-terminal (encoding B-box domain, 1–116 amino acids (aa)) and C-terminal (encoding CCT domain, 277–407 aa) parts were cloned into pGBKT7 as prey. The full-length coding sequence of *DTH8* was cloned into the pGADT7 vector and used as a bait. These constructs were cotransformed into yeast Y187 cells. Interactions between the bait and prey were observed on -3 Synthetic Dropout medium lacking -Leu-Trp-His, -4 Synthetic Dropout medium lacking Leu-Trp-His-Ade. Plates were incubated for 4 d at 30°C.

### Yeast one-hybrid (Y1H)

The 2.8 kb promoter sequence of *Ghd7* was divided into 10 fragments and cloned into the pAbAi vector; these were transferred into the yeast Y1H Gold strain. The *Hd1* sequence cloned in pGADT7 was used to transform Y1H Gold that contained the pAbAi constructs with the *Ghd7* promoter fragments. Positive interactions were confirmed by Y1H Gold yeast grown on Synthetic Dropout -Ura medium at 30 °C.

The primers used are listed in Supporting Information Table S3.

## Results

### *Hd1* is the factor suppressing heading under LD conditions

To study the molecular genetic basis of PS for heading in rice, we generated a rice recombinant inbred line population derived from a cross between an *indica* landrace (accession no. I7 with very strong PS) and another *indica* variety Qinghuazhan (with weak PS). From this population we identified an  $F_5$  family that segregated in a 3 : 1 ratio ( $\chi^2 = 0.24$ ,  $P > 0.5$ ) for dominant late-heading (153 plants) and recessive early-heading (47 plants) phenotypes, grown in Guangzhou, China under NLD conditions (in early season) (Fig. S1). When this segregating population was grown in sustained ALD conditions, the dominant plants (and the parent I7) showed a nonheading phenotype even after growing for > 250 d, while the recessive plants headed at 115 d. We named this dominant phenotype *NonHeading in LD (NHLD)*, and the recessive phenotype *nblnd*. When grown in the late season

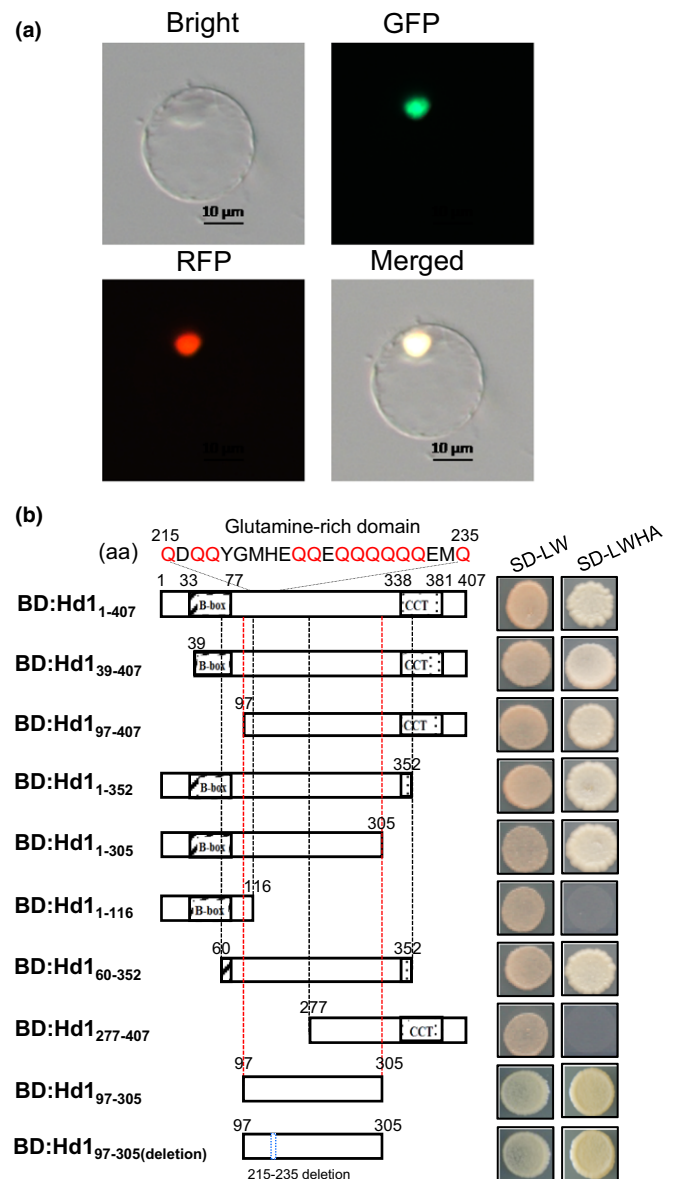
under NSD conditions (Fig. S1), however, the *NHLD* and *nhld* plants headed at 75.6 and 82.9 d, respectively (Fig. S2a,b). These observations indicated that the segregation of PS heading in this population is controlled by a single Mendelian genetic factor.

Genetic mapping using the segregating families (F<sub>5</sub> and F<sub>6</sub>) grown in ALD conditions narrowed down the locus controlling the *NHLD* and *nhld* phenotypes to a region on chromosome 6, where the heading date gene *Hd1* is located (Fig. S2c). Sequencing analysis indicated that *NHLD* plants (and I7) carry a functional allele, *Hd1-I7*, but *nhld* plants (and Qinghuazhan) have a mutant allele, *hd1*, with a 4 bp deletion in the second exon, which results in disruption of the conserved CCT domain of the protein (Fig. S2c). We prepared two binary constructs carrying the genomic fragments of *Hd1* alleles from I7 (*Hd1-I7*) and a *japonica* variety (Nipponbare) (*Hd1-Nip*), which has a 36 bp deletion in the first exon (see later) and introduced them into the *nhld* plants. Under ALD conditions, the *Hd1-I7*-containing transgenic (T<sub>2</sub>) lines did not flower even when grown for > 250 d (Fig. S2d,e). In NSD conditions, these T<sub>2</sub> lines headed at 103.3 d as compared with 83.5 d in the *nhld* plants. We also observed that the *Hd1-Nip*-containing transgenic T<sub>2</sub> line grown in ALD conditions headed at 141.9 d, 31 d later than the *nhld* plants (Fig. S2d,e). These results indicate that the *Hd1-I7* allele acts as a strong flowering repressor under LD conditions, and that *Hd1-Nip* is a weaker allele.

### Hd1 is a nuclear protein with transcriptional activation activity

Hd1 contains two B-box motifs and a CCT motif, which are highly conserved in plants and predicted to be involved in protein–protein interactions (Takahashi *et al.*, 2009; Gangappa & Botto, 2014). However, the protein characterization of Hd1 is still largely unknown. We investigated the subcellular localization of Hd1 through fusing the full-length coding sequence of *Hd1-I7* to the 5'-end of the enhanced green fluorescent protein gene (*eGFP*) driven by the CaMV 35S promoter. The transiently expressed Hd1:eGFP fusion protein in rice protoplasts colocalized with the nuclear marker TDR:RFP (Ji *et al.*, 2013), indicating that Hd1 is a nuclear protein (Fig. 1a). This result is consistent with a previous report that transient expression of Hd1 was accumulated in the nucleus of tobacco epidermal cells (Goretti *et al.*, 2017).

CO is a transcription factor, which has a glutamine-rich activation domain located between the B-box and CCT motifs (Tiawari *et al.*, 2003, 2010). Hd1 also contains a glutamine-rich region from aa 215–235 (Fig. 1b). We used a Y2H system to test whether this region has activation activity by fusing Hd1 and a set of deletion fragments to the GAL4-binding domain. The assay showed that Hd1 has strong transcriptional activation activity in yeast, and a region from 97 to 305 aa, but not including the B-boxes or the CCT domain, is essential for the activation activity (Fig. 1b). The 97–305 aa region is still active after deleting the glutamine-rich region (215–235 aa). These results suggest that Hd1 may activate the expression of downstream target gene(s) through its 97–305 aa region.



**Fig. 1** Characterization of Hd1 protein. (a) Subcellular localization of Hd1-eGFP fusion protein in rice protoplasts. The TDR-RFP fusion protein served as the nuclear marker. Bars, 10 μm. (b) Transcriptional activation assays of Hd1 and its truncated derivatives in the yeast GAL4 system. BD is the GAL4-DNA-binding domain. The red Q indicates glutamine (Gln). SD-LW, SD-Leu-Trp. SD-LWHA, SD-Leu-Trp-His + Ade.

### Genetic interactions among and between *Hd1*, *Ghd7* and *DTH8* determine differential PS

To test whether other heading-date regulatory genes contribute to the differential PS between *NHLD* and *nhld* plants, we sequenced some heading date genes. *NHLD* and *nhld* plants harbor functional alleles of *Ghd7*, *DTH8*, *Hd18*, *Ehd1*, *Hd3a* and *RFT1* (identical to the alleles in Nipponbare; Kojima *et al.*, 2002; Doi *et al.*, 2004; Zhao *et al.*, 2015; Zhang *et al.*, 2015; Shibaya *et al.*, 2016); functional *PRR37* (identical to the alleles in MH63; Gao *et al.*, 2014); functional *DTH3* (identical to the alleles in Dianjingyou 1, Bian *et al.*, 2011); and weakly functional *Ehd4* (identical to the alleles in 93-11; Gao *et al.*, 2013). These results

confirmed that except for the difference in the *Hd1* and *hd1* alleles, *NHLD* and *nhld* plants are homozygous for most (if not all) of the other major heading-date genes, consistent with the single-factor segregation for PS heading in this population.

*Ghd7* and *DTH8* are important suppressors of LD flowering. *Hd1* is reported to interact with *Ghd7* and *DTH8* at both the genetic level and the molecular level, and the complexes downregulate the expression of *Ehd1* and/or *Hd3a/RFT1* (Xue *et al.*, 2008; Wei *et al.*, 2010; Nemoto *et al.*, 2016; Du *et al.*, 2017). To systematically analyze the genetic relationship between and among these three genes in controlling differential PS in the same genetic background, we then used the CRISPR/CRISPR-associated protein 9 (Cas9) genome editing system (Ma *et al.*, 2015) to knock out *Hd1*, *Ghd7* and *DTH8* in *NHLD* plants. We obtained a number of knockout plants for these genes, and identified several homozygous loss-of-function mutant lines for each gene (Figs S3a–c, S4a,b). As expected, under ALD conditions the *Hd1*-knockout plants showed an early-heading phenotype (at 109.4 d), similar to that (at 113.2 d) of *nhld* plants. Under NSD conditions the heading dates of *Hd1*-knockout and *NHLD* plants were similar (at 78.6 and 77.2 d, respectively). Similarly, under ALD conditions, the *DTH8*-knockout plants headed at 132.0 d, and in NSD conditions they headed at 69.7 d. We also observed an early-heading phenotype (90.2 and 63.0 d in ALD and NSD conditions, respectively) for the *Ghd7*-knockout plants; this is notably earlier than those of *NHLD*, *nhld*, *Hd1*-knockout plants and *DTH8*-knockout plants in the corresponding day-length conditions (Fig. S4a,b). These results demonstrated that *Hd1*, *Ghd7* and *DTH8* are all required for the very strong PS in the *NHLD* (or I7) background, as loss of function of any one of these genes negates this PS.

Furthermore, we made several crosses with the *NHLD* line (with a genotype of *Hd1Hd1/Ghd7Ghd7/DTH8DTH8*, for short *HGD*), a *Hd1*-knockout line *hd1-6* (*bGD*), a *Ghd7*-knockout line *ghd7-2* (*HgD*), and a *DTH8*-knockout line *dth8-3* (*HGD*) to generate various combinations (eight in total) of double and triple mutant lines in the same genetic background: the wild-type *NHLD* line (*HGD-1*), single mutant lines (*bGD-2*, *HgD-3* and *HGD-4*), double mutant lines (*bGd-5*, *hgD-6* and *Hgd-7*) and a triple mutant line (*hgd-8*). Then we examined their heading date (and expression; see later) under ASD, NSD and ALD conditions during the same late season in Guangzhou.

We found that under ALD conditions, three lines carrying the functional *Ghd7* allele with a combination of functional or nonfunctional *Hd1* and/or *DTH8* (*HGD-1*, *bGD-2* and *HGD-4*) showed obviously delayed heading as compared with the other lines (Fig. 2a,b). When all eight lines were grown under ASD conditions, four lines carrying functional *Hd1* (*Hgd-7*, *HgD-3*, *HGD-1* and *HGD-4*) headed earlier than the *hd1*-carrying lines (*bGD-2*, *bGd-5*, *hgD-6* and *hgd-8*) (Fig. 2a,b). These observations imply that *Ghd7* is the major heading-repressor gene under LD conditions but it requires *Hd1* and/or *DTH8*, while *Hd1* is the heading-promoter gene under SD conditions.

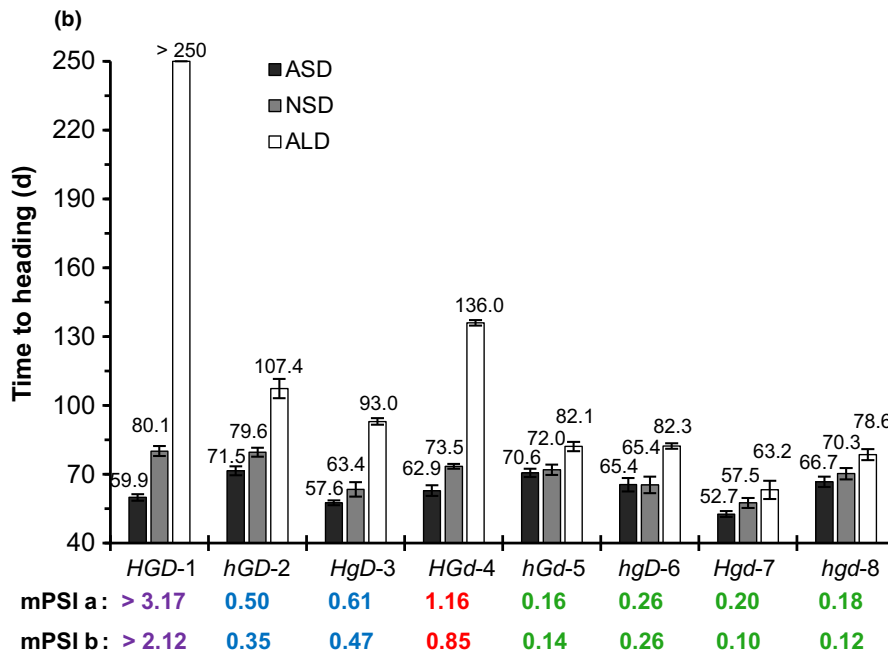
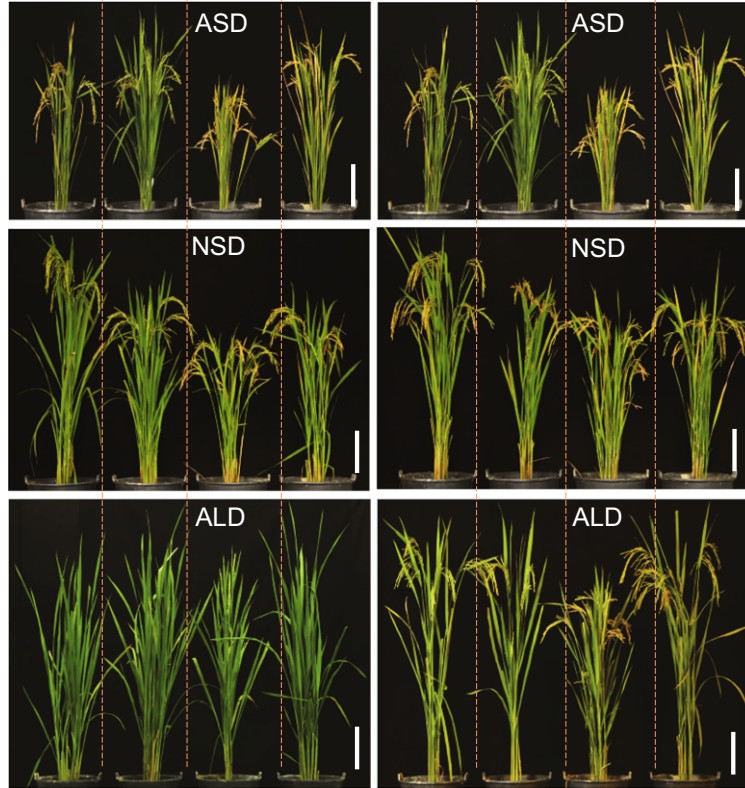
The PS index (PSI), previously defined as  $PSI = [DTH_{LD} - DTH_{SD}]/DTH_{LD}$  ( $DTH_{LD}$  and  $DTH_{SD}$  represent days to heading under LD and SD conditions, respectively), is an indicator for quantitatively measuring PS in rice (Immark *et al.*, 1997). In this study, we observed the heading date of these lines grown in the same period under ASD, NSD and ALD conditions. Since *NHLD* plants (*HGD-1*) were nonheading in ALD conditions, the previous PSI definition is not suitable for this genotype. Therefore, we used modified PSI formulas ( $mPSIa = (DTH_{ALDc} - DTH_{ASD})/DTH_{ASD}$ , or  $mPSIb = (DTH_{ALD} - DTH_{NSD})/DTH_{NSD}$ ) in this study.

*HGD-1* plants had the highest mPSI (> 3.17 based on ASD conditions or > 2.12 based on NSD conditions), *HGD-4* plants had a relatively higher mPSI (1.16 or 0.85), and two lines (*bGD-2* and *HgD-3*) exhibited moderate mPSI values (0.50 and 0.61, respectively) (Fig. 2b). The lines carrying one or none of these functional genes (*bGd-5*, *hgD-6*, *Hgd-7* and *hgd-8*) had low mPSI values (0.16, 0.26, 0.20 and 0.18, respectively). The finding of the *hgd-8* triple mutant having a low mPSI value suggests that other photoperiod-related heading date gene(s), if any, can only weakly affect PS independent of *Hd1*, *Ghd7* and *DTH8*.

We calculated the net genetic effect and carried out significance analysis to quantify the monogenic, digenic and trigenic effects among *Hd1*, *Ghd7* and *DTH8* on heading date under different day-lengths. We first analyzed the genetic effect of single genes in regulating heading. We found that in the *ghd7/dth8* background (*Hgd-7* vs *hgd-8*), *Hd1* promoted heading by 14 and 15 d in ASD and ALD conditions, respectively, with significant net genetic effect (*e*) on heading (Fig. 2b; Table 1), while in the *hd1/dth8* background (*bGd-5* vs *hgd-8*), *Ghd7* suppressed heading by 4 d in both ASD and ALD conditions (Fig. 2b, Table 1). In the *hd1/ghd7* background (*hgD-6* vs *hgd-8*), *DTH8* also slightly suppressed heading (by 4 d) under ALD conditions (Fig. 2b; Table 1).

Next, we evaluated the effects of two-gene combinations and compared them to the effects of single genes. Under ALD conditions, the *Hd1/Ghd7* combination (*HGD-4*) showed relatively stronger heading suppression effect (delayed heading by 54 d as compared with the *bGd-5* line). Under ASD conditions, the heading date of the *HGD-4* line was 8 d earlier than that of the *bGd-5* line, but 10 d later than that of the *Hgd-7* line (Fig. 2b). Under ALD conditions the suppression genetic effect of the *Hd1-DTH8* interaction (in *HgD-3*) was moderate (delayed by 30 d) as compared with *Hgd-7*, and was weak (delayed heading by 10 d) when compared with the *hgD-6* line. The *Hd1/Ghd7* and *Hd1/DTH8* combinations were detected with significant digenic genetic effects (as compared with the responding single gene effects) in ALD, NSD and ASD conditions; for example, *Hd1/Ghd7* had an *e*-value of 69.3 under ALD conditions (Table 1). The *Ghd7-DTH8* interaction in *bGD-2* also had a moderate suppression effect (delayed heading by 25 d) under ALD conditions as compared with the *bGd-5* and *hgD-6* lines (with an *e*-value of 21.6), respectively (Fig. 2b; Table 1), confirming the presence of high-order genetic interaction among the three genes, in addition to the accumulative effects of single gene and double-gene combinations.

(a) *HGD-1* *hGD-2* *HgD-3* *HGd-4* *hGd-5* *hgD-6* *Hgd-7* *hgd-8*



**Fig. 2** Eight isogenic lines with combinations of the *Hd1*, *Ghd7* and *DTH8* alleles generated by gene knockout and crossing. (a) Phenotypes of the lines (97-d-old plants) under artificial short-day (ASD), natural short-day (NSD) and artificial long-day (ALD) conditions in Guangzhou. Bars, 25 cm. In *NHLD* background (*HGD-1*), which has strong photoperiod sensitivity (PS), *Hd1*, *Ghd7* and *DTH8* were knocked out, respectively, via CRISPR/Cas9 to generate mutant lines (*hGD-2*, *HgD-3*, *HGd-4*). Then a set of eight isogenic lines with all combinations of *Hd1*, *Ghd7* and *DTH8* alleles were developed by crossing among *hGD-2*, *HgD-3* and *HGd-4* to generate *hGd-5*, *hgD-6*, *Hgd-7* and *hgd-8*". (b) Heading dates of the lines in the different day-length conditions, and their modified PS index (mPSI) values based on ASD (a) or NSD conditions (b). Data are means  $\pm$  SD ( $n = 50$ ). The degrees of PS are classified into very strong (purple), strong (red), moderate (blue), and weak (green) based on the mPSI values.

**Table 1** Net genetic effect (*e*) on heading date of single genes and their combinatorial effects of *Hd1*, *Ghd7* and *DTH8* in different day-length conditions.

| Lines        | Monogenic <i>e</i> |         |         | Digenic <i>e</i> |         |         | Trigenic <i>e</i> |         |           |
|--------------|--------------------|---------|---------|------------------|---------|---------|-------------------|---------|-----------|
|              | ASD                | NSD     | ALD     | ASD              | NSD     | ALD     | ASD               | NSD     | ALD       |
| <i>Hgd-7</i> | -14.1**            | -12.8** | -15.4** |                  |         |         |                   |         |           |
| <i>hGd-5</i> | +3.8**             | +1.7**  | +3.5**  |                  |         |         |                   |         |           |
| <i>hgd-6</i> | -1.3*              | -4.9**  | +3.7**  |                  |         |         |                   |         |           |
| <i>HGD-4</i> |                    |         |         | +6.3**           | +14.2** | +69.3** |                   |         |           |
| <i>Hgd-3</i> |                    |         |         | +6.2**           | +10.8** | +26.1** |                   |         |           |
| <i>hGD-2</i> |                    |         |         | +2.2*            | +12.5** | +21.6** |                   |         |           |
| <i>HGD-1</i> |                    |         |         |                  |         |         | -10.0**           | -11.8** | > +62.7** |

ASD, artificial short-day conditions; ALD, artificial long-day conditions; NSD, natural short-day conditions.

'-' indicates the promotion effect on heading from functional gene. '+' indicates the inhibition effect on heading from functional gene. \*, \*\*, significance at 5% and 1% levels, respectively.

The single-gene effects, for example, the effect of *Hd1*, were calculated by subtracting the basic effect of the *hgd* genotype by  $e_{(H)} = D_{(Hgd-7)} - D_{(hgd-8)}$ , to clarify the original function of *Hd1*.

The double-gene effects, the effect of the *Hd1/Ghd7* combination, were calculated by subtracting the effects of the *hgd* genotype and the single-gene effects,  $e_{(HGd)} = D_{(HGd-4)} - D_{(hgd-8)} - e_{(H)} - e_{(G)}$ .

The effect of the *Hd1/Ghd7/DTH8* combination, the basic effect of the *hgd* genotype, the single-gene effects, and the double-gene effects are all subtracted,  $e_{(HGD)} = D_{(HGD-1)} - D_{(hgd-8)} - e_{(H)} - e_{(G)} - e_{(D)} - e_{(HGd)} - e_{(HGD)} - e_{(HGD)}$ .

We noticed that the presence of the functional *Ghd7* or *DTH8* alleles changed the primary function of *Hd1* from promotion to suppression of heading under ALD conditions, while in the ASD condition, the promotion effect of *Hd1* alone (*Hgd-7* vs. *hgd-8*) with 14 d was competed and partially impaired by *Ghd7* (in *HGD-4*) or *DTH8* (in *HgD-3*), leading to only promoted heading for 8 d, as *HGD-4*, *HgD-3* compared to the *hGd-5*, *hgd-6* respectively (Fig. 2b; Table 1). Here, we confirmed again that the *Hd1-Ghd7-DTH8* trigenic interaction in *HGD-1* produced the very strong inhibition effect (with an *e*-value > 62.7) under ALD conditions (Fig. 2b; Table 1).

Taken together, our results suggest that *Hd1* promotes flowering and *Ghd7* represses flowering regardless of day-length. The *Ghd7/DTH8* combination can enhance the suppression under LD conditions. The *Hd1/DTH8*, *Hd1/Ghd7* and *Hd1/Ghd7/DTH8* combinations have incrementally enhanced LD-suppression roles that convert the *Hd1* effect from promotion to great delays of heading, but they compete the promotion effect of *Hd1* to different extents in SD conditions, which determine the degrees of PS in rice.

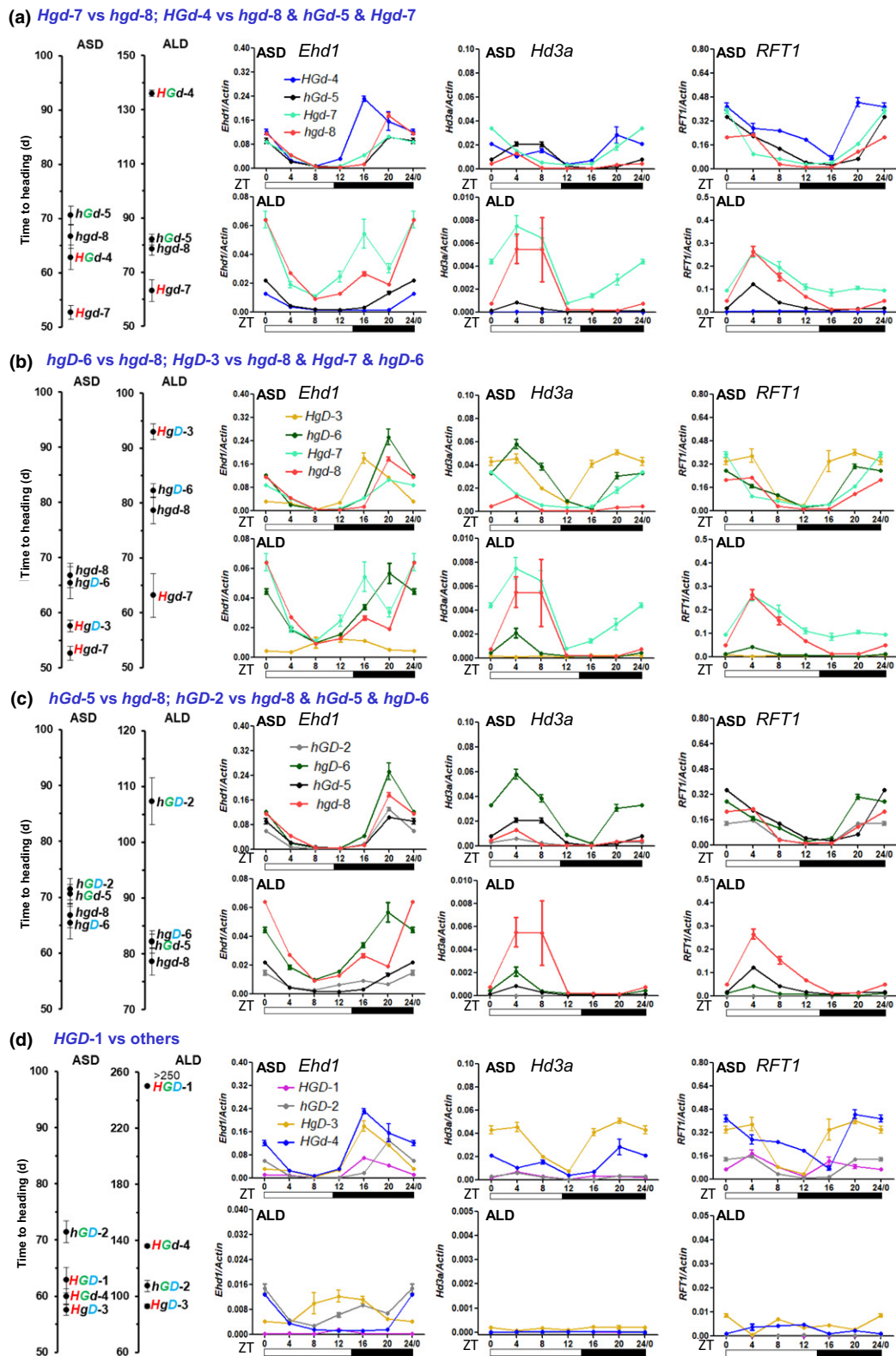
### *Hd1*, *Ghd7* and *DTH8* regulate *Ehd1* and *Hd3a/RFT1* expression in a complex manner

*Ehd1* is the essential integrator gene that regulates the expression of the florigen genes *Hd3a* and *RFT1* under different day-lengths (Zhao *et al.*, 2015). We analyzed their expression in the eight lines discussed earlier, to understand the molecular effects of *Hd1*, *Ghd7* and *DTH8* and their combinations. We found that the overall expression levels of *Ehd1*, *Hd3a* and *RFT1* in these lines were roughly consistent with their heading dates in the ALD and ASD conditions (Fig. S5a,b), showing significant Pearson's correlation coefficients between the heading date and gene expression level under ALD conditions at most of the time-points (Table S1). We also compared the expression of *Ehd1*, *Hd3a* and

*RFT1* under ALD and ASD conditions in the eight lines. The results showed that the lines with high mPSI values had larger differences in the expression of these genes between the ALD and ASD conditions. For the *hgd-7* and *hgd-8* lines with weak PSI values, the RNA levels of *Ehd1*, *Hd3a* and *RFT1* were very similar (Fig. S6a-h).

We then analyzed the regulation of *Ehd1*, *Hd3a* and *RFT1* expression in the *hGd-5*, *hgd-6* and *Hgd-7* lines. Under both ASD and ALD conditions, the expression levels of *Hd3a* and *RFT1* in the *Hgd-7* line, mainly at Zeitgeber time 20 (ZT20) and ZT24, were higher than in the *hgd-8* line (Fig. 3a). In the *hd1/ghd7* background (*hgd-6* vs *hgd-8*), *DTH8* slightly suppressed heading in ALD by repressing the expression of *Ehd1*, *Hd3a* and *RFT1* in the morning (Fig. 3b). In the *hd1/dth8* background (*hGd-5* vs *hgd-8*), *Ghd7* weakly suppressed heading in ALD by repressing the expression of the *Ehd1-Hd3a/RFT1* pathway from dawn to morning. While under ASD conditions, *Ghd7* also weakly suppressed heading, but the transcript abundances of *Ehd1/RFT1/Hd3a* were similar to those of *hgd-8* (Fig. 3c).

We then deduced the interactive effects of the two functional-allele combinations. For the *Hd1/Ghd7* combination (in *HGD-4*), under ASD conditions, it showed higher levels of *Ehd1* and *Hd3a/RFT1* expression than the *hgd-8* line and earlier heading, while under ALD conditions the expression of the *Ehd1-Hd3a/RFT1* pathway was strongly suppressed mainly at ZT20 and ZT24 compared with *hgd-8* and *hGd-5*, leading to very late heading (Fig. 3a). The genetic effect of the *Hd1/DTH8* combination (*HgD-3* vs *hgd-8*) was similar to that of the *Hd1/Ghd7* combination (*HGD-4*) in the ALD and ASD conditions (Fig. 3b). Under ALD conditions the *Ghd7/DTH8* combination (*hGD-2* vs *hgd-8* and *hGd-5*) showed stronger suppression effects to the *Ehd1-Hd3a/RFT1* pathway in the morning, but under ASD conditions it showed only weak suppression to this pathway (Fig. 3c). We again confirmed that the combination of *Hd1/Ghd7/DTH8* (*HGD-1*) completely suppressed the *Ehd1-Hd3a/RFT1* pathway



**Fig. 3** Heading dates and expression of *Ehd1*, *Hd3a* and *RFT1* in the eight isogenic lines under artificial short-day (ASD) and artificial long-day (ALD) conditions. Fifty-eight-day-old plants were analyzed. (a) Comparisons of the effects with and without *Hd1* (*Hgd-7* vs *hgd-8*), and with and without the *Hd1-Ghd7* genetic interaction (*HGD-4* vs *hgd-8* & *hGd-5* & *Hgd-7*). (b) Comparisons of the effects with and without *DTH8* (*hgd-6* vs *hgd-8*), and with and without the *Hd1-DTH8* genetic interaction (*HGD-3* vs *hgd-8* & *Hgd-7* & *hgd-6*). (c) Comparisons of the effects with and without *Ghd7* (*hGd-5* vs *hgd-8*), and with and without the *Ghd7-DTH8* genetic interaction (*hGD-2* vs *hgd-8* & *hGd-5* & *hgd-6*). (d) Comparisons of the effects with and without the *Hd1-Ghd7-DTH8* genetic interaction (*HGD-1* vs others). ZT, Zeitgeber time. Error bars represent SD of three biological replicates.



at all time-points under ALD conditions, but moderately repressed this pathway under ASD conditions (Fig. 3d). These data support the idea that the primary function of *Ghd7* is to suppress the expression of the *Ehd1-Hd3a/RFT1* pathway, whereas the primary function of *Hd1* is to promote *Hd3a/RFT1* expression, regardless of day-length. In LD conditions, the *Ehd1-Hd3a/RFT1* pathway is progressively suppressed by these combinations – *Hd1/DTH8*, *Ghd7/DTH8*, *Hd1/Ghd7* and *Hd1/Ghd7/DTH8* – while in SD conditions, the expression patterns of *Ehd1* and *Hd3a/RFT1* are regulated in a more complex manner.

We also analyzed the expression of *Hd1*, *Ghd7* and *DTH8* in these lines and explored their possible regulatory relationships based on their genetic effects. We observed that under ASD conditions, the expression levels of *Ghd7* in the *HGD-1*, *HGD-2*, *hGD-4* and *hGD-5* lines were very low, but the *Ghd7* mRNA levels were increased in the daytime under ALD conditions. This is consistent with the notion that *Ghd7* exerts a repressive effect mainly in LD conditions. Under ALD conditions, the *Ghd7* mRNA levels at ZT4 in the lines with *Hd1* (*HGD-1* and *HGD-2*) were higher than those in the *hGD-4* and *hGD-5* lines (Fig. S7a). *DTH8* has peak expression during the darkness in ASD, and higher expression during the dusk and darkness in ALD conditions (Fig. S7b). In ASD conditions, the *Hd1* mRNA levels in the *HGD-1*, *HGD-3*, *HGD-4* and *HGD-7* lines were similar, but under ALD conditions the *Hd1* mRNA levels in the *HGD-1* and *HGD-4* lines were higher than those in the *HGD-3* and *HGD-7* lines (Fig. S7c). Considering that the *Hd1/Ghd7* and *Hd1/Ghd7/DTH8* genotypes have stronger repression of flowering in LD conditions, we speculated that *Hd1* might play a role in promoting *Ghd7* expression, thus enhancing the function of *Ghd7*. Indeed, using Y1H assays we detected direct binding of the Hd1 protein to the promoter region of *Ghd7* (Fig. S8a,b), supporting the presence of a protein–DNA interaction between these two genes.

### Hd1, Ghd7 and DTH8 form functional complexes

Previous reports showed that pairwise interactions exist between Hd1, Ghd7 and DTH8. (Nemoto *et al.*, 2016; Du *et al.*, 2017; Goretti *et al.*, 2017; Cai *et al.*, 2019). In this study we also detected their interactions using different methods. GST-tagged Ghd7 could bind MBP-tagged Hd1 (Fig. 4a), MBP-tagged DTH8 could pull down HIS-tagged Hd1 (Fig. 4b), and GST-tagged Ghd7 could pull down MBP-tagged DTH8 (Fig. 4c). Y2H assays also confirmed that DTH8 interacted with both the B-box and the CCT domain of Hd1, and BiFC assay verified that Ghd7-YFP<sup>n</sup> and DTH8-YFP<sup>c</sup> interacted in the nuclei of rice protoplasts (Fig. S9a,b). Finally, we further used a three-protein pulldown assay to verify that MBP-Hd1 and MBP-DTH8 were simultaneously pulled down by GST-Ghd7 (Fig. 4d). Moreover, in this experiment regardless of the binding prioritization of Hd1 and DTH8 by sequentially or simultaneously adding the proteins to the reactions, they could equally bind to Ghd7 without biased competition (Fig. 4d), indicating that the three proteins interacted with each other through different binding domains to form a complex (the HGD complex).

### Natural variations of *Hd1*, *Ghd7* and *DTH8* confer PS evolution

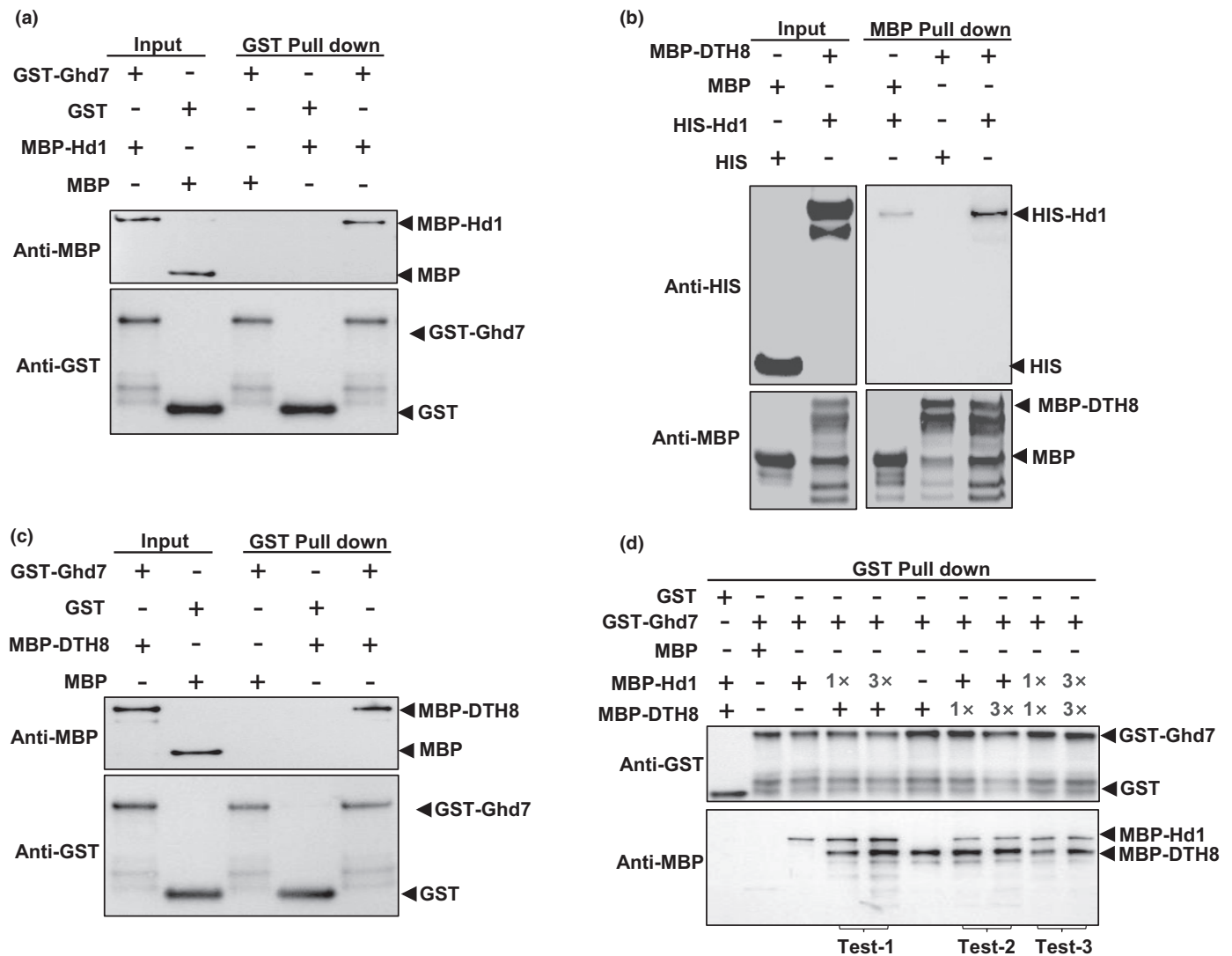
To explore the relationship between the natural variation of these genes and PS evolution during rice domestication/breeding, we sequenced the coding region of *Hd1*, *Ghd7* and *DTH8* from a germplasm collection, including 65 landraces (native cultivars), 61 modern varieties, and an *O. rufipogon* accession, and classified these genes into functional and nonfunctional haplotypes based on the prediction of deleterious effect of amino acid changes through the SIFT website. We also observed the heading dates of these materials in the same late season under ASD (or NSD) and ALD conditions, or under early season NLD and late season in Guangzhou (Table S2). Then we analyzed the relationship between the different combinations of the gene types and the degree of PS.

Thirteen *Hd1* haplotypes (types) were detected in these cultivated rice lines (and *O. rufipogon*); seven types are functional and six types are nonfunctional. For *Ghd7*, 17 haplotypes were detected among these materials, of which two (types 15 and 16) are nonfunctional. Nine haplotypes of *DTH8* were identified and five of them are functional (Fig. S10a–c; Table S2). These functional haplotypes might include normal- or reduced-function variations, such as the weakly functional type 14 present in Nipponbare verified by genetic transformation (Fig. S2d,e).

The perennial wild rice *O. rufipogon* contains functional alleles of *Hd1* (Type 13), *Ghd7* (type 17) and *DTH8* (type 5) (Fig. S10d); this genotype confers very strong PS (e.g. heading only in October in typical SD conditions). Among the 65 landraces, which are mainly distributed in subtropical regions, 41 lines had the *HGD*-type combinations, and most of them (36 lines) showed strong or very strong PS (no-heading in ALD conditions). The other 21 lines had non-*HGD*-type combinations with weak or even no PS (Fig. 5a,c; Table S2). Strikingly, most of the modern *indica* varieties cultivated in South China were the *hGD*-type with low mPSI values (Fig. 5a,c; Table S2), suggesting that weak PS had been selected in breeding of the modern *indica* rice varieties. In the modern *japonica* rice varieties, those (11 varieties) mainly cultivated in Middle China contained the *HGD*-type combination and exhibited relatively strong PS, while those with other genotypes producing weak PS are cultivated in North and North-east China (Fig. 5b; Table S2).

### Discussion

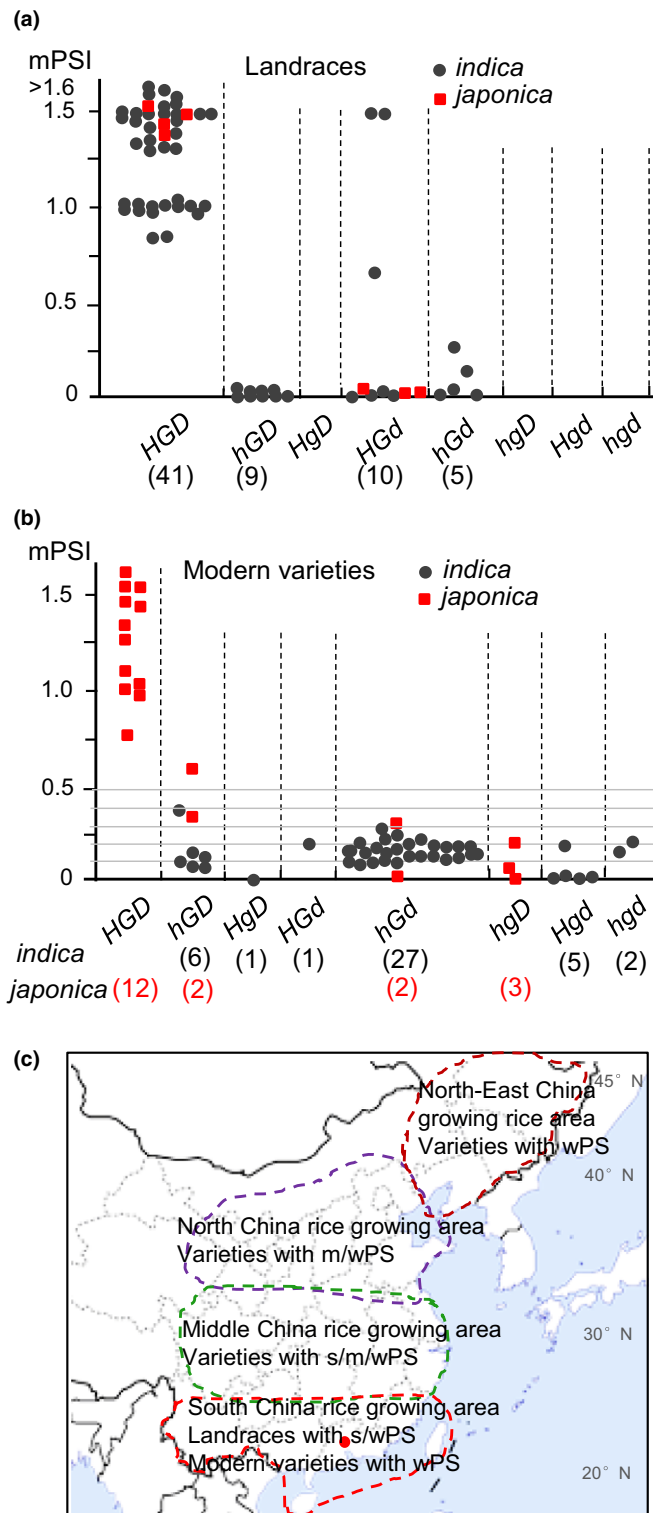
In this study, we systematically analyzed the genetic effects and the regulatory mechanism of the *Hd1*, *Ghd7* and *DTH8* alleles and their combinations for controlling PS variation in rice by using a set of eight isogenic lines with single, dual or triple knockout of these genes, and a collection of landraces and modern cultivars. Based on the results, we proposed a molecular model of this regulatory system (Fig. 6a,b). *Hd1* has a primary function for promoting the expression of *Hd3a/RFT1* and heading regardless of the day-length, while *Ghd7*



**Fig. 4** *In vitro* pulldown assays for interactions between and among Hd1, Ghd7 and DTH8. (a) Hd1 interacted with Ghd7. GST-Ghd7 is used as bait. (b) Hd1 interacted with DTH8. MBP-DTH8 is used as bait. (c) DTH8 interacted with Ghd7. GST-Ghd7 is used as bait. (d) Interactions among Hd1, Ghd7 and DTH8. GST-Ghd7 was used as a bait. In Test-1, the prey MBP-Hd1 protein was first added to the bait GST-Ghd7. From 1× to 3×, the intensity of the anti-MBP hybridization band of MBP-Hd1 gradually increased, indicating that more MBP-Hd1 was pulled down by GST-Ghd7. After washing, the second prey, MBP-DTH8, was added, but the amount was constant. The intensity of the hybridization band of MBP-DTH8 was also increased, which implies that DTH8 was more likely to bind the increased Hd1, proving the formation of a three-protein complex of Ghd7/Hd1/DTH8. In Test-2, the prey MBP-DTH8 was added first to the bait GST-Ghd7. From 1× to 3×, the anti-MBP hybridization band of MBP-DTH8 remained unchanged, indicating that 1× of DTH8 was enough to bind to all the bait GST-Ghd7. After washing, the second prey MBP-Hd1 was added with a constant amount. Its hybridization band was basically unchanged, implying that Hd1 bound to Ghd7 and DTH8 with a different domain, forming a three-protein complex. In Test-3, 1× of prey MBP-DTH8 and MBP-Hd1, or 3× of prey MBP-DTH8 and MBP-Hd1 were added simultaneously to the reaction. It was observed that the hybridization bands of MBP-DTH8 and MBP-Hd1 had similar intensities. Test-1, Test-2 and Test-3 together explained that Ghd7/DTH8/Hd1 formed a three-protein complex at a ratio of 1 : 1 : 1, instead of independent pairwise interactions. It is not the case that the amount of GST-Ghd7 was excessive, and thus a part of it bound to MBP-Hd1 and another part of it interacted with MBP-DTH8.

(*hGd*) or *DTH8* (*hgD*) alone has only a weak effect on suppressing heading in LD conditions. However, in the lines with different combinations of *Hd1*, *Ghd7* and *DTH8*, Hd1 is recruited by Ghd7 and/or DTH8 to form repressive complexes (Hd1/DTH8, Ghd7/DTH8, Hd1/Ghd7 or Hd1/Ghd7/DTH8), which possess gradually enhanced effects to suppress the *Ehd1-Hd3a/RFT1* pathway, thus repressing heading in increasing degrees. In SD conditions, the repressive functions

of these complexes decrease, and Hd1 plays its original promotion role to compete the repressive complexes. These characters exhibit a collaborative effect in LD conditions and an antagonistic effect in SD conditions between *Hd1* and *Ghd7/DTH8*. The genetic interactions of these alleles mediated by the opposing actions of *Hd1* enlarge the differences of heading date between LD and SD conditions, thus conferring various degrees of PS in rice populations (Fig. 6).



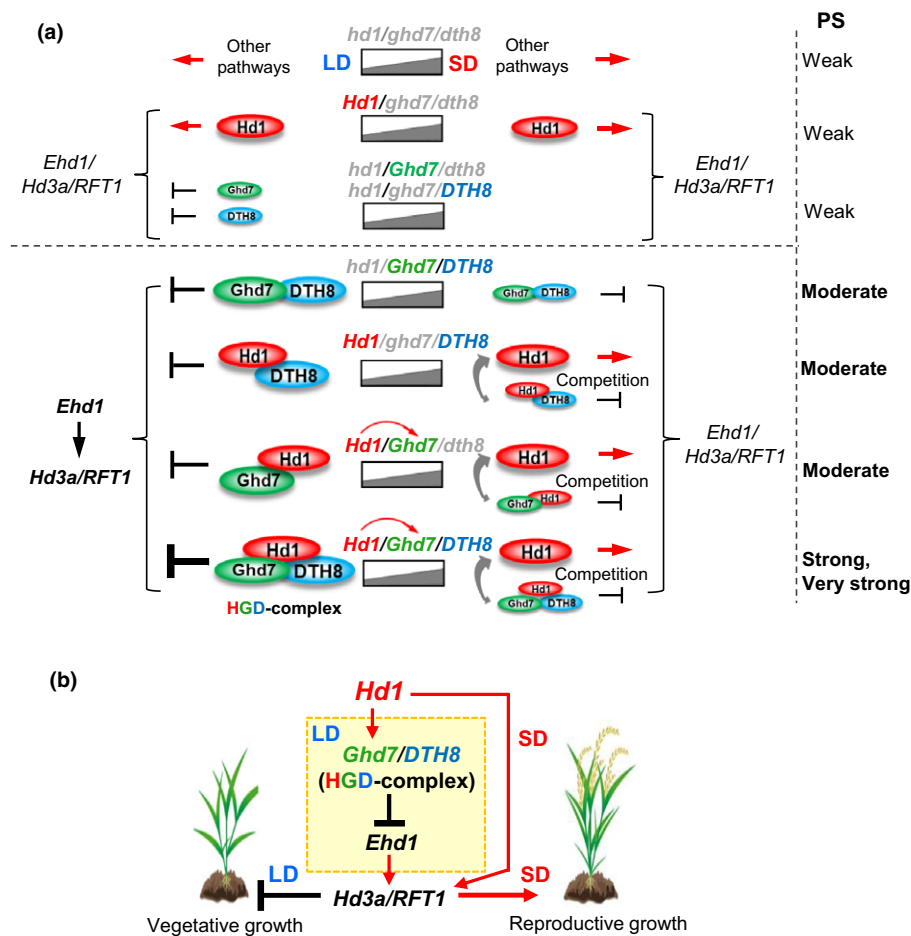
**Fig. 5** *Hd1*, *Ghd7* and *DTH8* allele combinations confer variations in rice photoperiod sensitivity (PS) and contribute to geographical adaptation. (a, b) Modified PS index (mPSI) of landraces (a) and modern *indica* varieties (b), which are mainly distributed in South China, and modern *japonica* varieties (b) in Middle and North/Northeast China. The functional types of the genes (*H*, *G*, *D*) include various haplotypes that may have normal- or reduced-function variations. The nonfunctional alleles (*h*, *g*, *d*) were derived from mutations causing frame-shift. The mPSI values in (a) were measured with heading dates under artificial long-day (ALD) and natural short-day (NSD) conditions, and those in (b) were based on heading dates under ALD and artificial short-day (ASD) conditions for *japonica* varieties and NLD and NSD conditions for *indica* varieties. (c) Adaptation of rice varieties to different day-lengths and cropping systems in the main rice-growing areas in China. In the South China (Guangzhou is indicated with a red dot) rice-growing area where mainly *indica* varieties are cultivated, those landraces (carrying the *HGD*-type) with strong photoperiod sensitivity (sPS) are planted in the late season (with SD conditions), and some landraces and most modern varieties (mainly containing the *hGd*-type) having weak PS (wPS) are planted in the early season (with LD conditions) and the late season. In Middle China rice-growing areas where both *indica* and *japonica* varieties are cultivated, *indica* varieties having wPS are planted in the early season (with LD conditions) and the late season (with SD conditions), or in the mid-season (with SD conditions, heading in late August to early September). And *japonica* varieties having sPS or moderate PS (mPS) (mainly containing the *HGD*-type or *hGD*-type) are planted in the mid-season (with SD conditions, heading in early September). In North and Northeast China rice-growing areas, only *japonica* varieties having wPS are planted in the mid-season (with LD conditions, heading in early to mid-August).

promotion and *HGD*-type repression reaches a balance: *Hd1* may neither promote nor repress heading (Fig. 2b). Second, why do *Ghd7* or *DTH8* have large differences of LD-suppression effects in different background? We revealed that the LD-suppression effect of *Ghd7* (*hGd*) or *DTH8* (*hgD*) alone is weak in plants lacking functional *Hd1*, but this effect is largely enhanced upon genetic interactions with *Hd1*. Third, we demonstrate that *Hd1*-*H3dalRFT1* and (*Ghd7/DTH8*)-*Ehd1*-*Hd3alRFT1* are not simply two independent pathways for controlling heading date as previously proposed (Song *et al.*, 2015). In fact, *Hd1* has bidirectional roles in mediating the crosstalk between the SD- and LD-related molecular pathways *Hd1*-*Hd3alRFT1* and (*Hd1/Ghd7/DTH8*)-*Ehd1*-*H3dalRFT1*, respectively (Fig. 6b).

Importantly, our results (Table 1) clearly indicate that the net genetic effect of the *HGD*-1 line under LD conditions (the genetic effect of the three genes after subtracting those of all the single genes and two-gene combinations) was much larger than zero (> 62.7 d), meaning that the genetic effect of the three genes is not simply an accumulative and replacing effect of the single genes and two-gene interactions, but there must be a high-order genetic/molecular interaction among the three (and possibly more other(s)) genes, which produces a much stronger LD-suppression effect than the total single- and two-gene effects.

Under LD conditions, the *Hd1/Ghd7/DTH8* and *Hd1/Ghd7* combinations completely and largely inhibited the *Ehd1-H3dalRFT1* pathway, respectively. We speculate that this is partly a result of the promoted *Ghd7* expression by *Hd1* (Fig. S7a). On the other hand, besides the expression regulation of the genes, the protein complexes with *Hd1*, *Ghd7* and/or *DTH8* may possess

Our results clarified several questions. First, why doesn't *Hd1* always promote flowering under SD conditions and suppress it under LD conditions. The primary functions of *Hd1*, in the *ghd7/dth8* background, is for promoting flowering, regardless of day-length. In the *HGD* lines, when the day-length is shorter than a certain threshold, the competition between the *Hd1*



**Fig. 6** A working model of the interactions among *Hd1*, *Ghd7* and *DTH8* for regulating heading date and photoperiod sensitivity (PS) in rice. (a) In the *hd1/ghd7/dth8* genotype, the basal expressions of *Ehd1* and *Hd3a/RFT1* are regulated by other pathways. '*Ehd1/Hd3a/RFT1*' means that expression of these genes is regulated independently by the upstream regulator(s), or *Ehd1* is first regulated and then *Hd3a/RFT1* are regulated by *Ehd1*, or both ways. In the absence of functional *Ghd7* and *DTH8* alleles, *Hd1* shows a primary role of promoting *Hd3a/RFT1* expressions and heading under both short-day (SD) and long-day (LD) conditions. *Ghd7* (*hGd*) or *DTH8* (*hGd*) alone have weak effect on suppressing heading, mainly in LD conditions. In various combinations among the *Hd1*, *Ghd7* and *DTH8* alleles, under LD conditions *Hd1* promotes *Ghd7* expression and is also recruited by *Ghd7* and/or *DTH8* to form repressive complexes that have differently enhanced suppression effects on the *Ehd1*-*Hd3a/RFT1* pathway for repressing heading. In SD conditions, owing to the weakened suppression effect of *Ghd7*, the repressive functions of these complexes are decreased, and *Hd1* competes with the complexes to promoting heading with reduced extents. The interactions of the different gene combinations (including multiple allelic variations of the genes) produce various extents of suppression and promotion effects on heading in response to different day-lengths, and thus control different heading dates and confer various degrees of PS in rice populations. The different sizes of the marks for the proteins and promotion and repression indicate their relatively different levels of effect. Notably, the HGD working model we proposed for the PS control is based on the presence of other possibly essential factor(s) that are involved in photoperiodic flowering regulation in rice. (b) Simplified rice flowering regulatory pathways mediated commonly by *Hd1* in LD and SD conditions. Other known factors related to these two pathways are omitted.

largely enhanced or new biochemical functions compared with the single proteins. *Hd1* and *Ghd7* are CCT-domain-containing proteins (Yano *et al.*, 2000; Xue *et al.*, 2008), while *DTH8* is a subunit of the NF-Y complex (Wei *et al.*, 2010). The NF-Y complex is a sequence-specific heterotrimeric transcription factor (Mantovani, 1999). Many CCT-domain proteins, which are involved in flowering regulation in plants, interact with NF-Y subunits to regulate transcription of target genes (Song *et al.*, 2015; Brambilla & Fornara, 2017). The *Ghd7/Hd1* complex can directly bind the *Ehd1* promoter (Nemoto *et al.*, 2016). Therefore, in LD conditions, *Hd1*, *Ghd7* and *DTH8* may form active NF-Y complexes that undergo dynamic changes in composition and/or protein modifications in response to different day-lengths.

In SD conditions, the expression of *Ghd7* is decreased and *Hd1* may form a complex with a blue light-responsive flavin mononucleotide-binding protein *OshAL3*, which binds to the promoter region of *Hd3a* (and possibly also to that of *RFT1*) for promoting its expression (Su *et al.*, 2016).

The *Arabidopsis* CO protein accumulates specifically in the daytime under LD conditions, which induces the expression of *FT* to promote flowering (Song *et al.*, 2012). CO accumulation is regulated by the GI/FKF1-CDF1 module, and the different effects of day-length on flowering (LD promotes flowering and SD has no effect) are determined by the coincident and staggered expression phases of GI and FKF1 in LD and SD conditions, respectively (Sawa *et al.*, 2007). We found that in rice, the CO

homolog *Hd1* promotes the expression of the *FT* orthologs *Hd3aRFT1* in both SD and LD conditions. This indicates that the basic *CO-FT* and *Hd1-Hd3aRFT1* regulatory pathways are functionally conserved in LD and SD plants, but rice has specifically evolved the (*Hd1/Ghd7/DTH8*)-*Ehd1-H3dalRFT1* pathway, which recruited the conserved Hd1 to a novel repressive function.

Moreover, the differential effects of day-length on rice heading are determined by the conflict between the SD-promotion *Hd1-Hd3aRFT1* pathway and the LD-repression (*Hd1/Ghd7/DTH8*)-*Ehd1-H3dalRFT1* pathway, indicating the genetic diversity of flowering-control mechanisms between monocot and dicot species. Homologous genes of *Hd1*, *Ghd7*, *DTH8* and *Ehd1* exist in maize (*Zea mays*) and sorghum (*Sorghum bicolor*) (Brambilla *et al.*, 2017), suggesting that the PS-flowering mechanism in rice may also be conserved in other tropical-origin monocot SD plants.

*Hd1*, *Ghd7* and *DTH8* show abundant natural variations (Fig. S10; Table S2), which greatly increased the genetic complexity in heading-date control and PS in rice. We show that most rice landraces, like *O. rufipogon*, contain functional *Hd1*, *Ghd7* and *DTH8* alleles and have strong or very strong PS. In the past, such landraces could only be cultivated in NSD conditions in tropical and subtropical regions. Our results demonstrate that the evolution of rice PS from very strong in the wild rice and landraces to various reduced degrees in the modern varieties during rice domestication and breeding mainly involved natural allelic variations (including functional and nonfunctional haplotypes) of *Hd1*, *Ghd7* and *DTH8* and their genetic interactions (Figs 5, S10). For example, many modern *indica* varieties were bred to possess the *bGd*-type and *bGD*-type of the genes, having low sensitivity to day-length (Fig. 5b), which are suitable for broad cultivation in both NLD and NSD conditions in South China and Middle China areas with different day-length conditions. The modern *japonica* varieties with strong or moderate PS (mainly having the *HGD*-type) are suitable for planting in the mid-season (single-cropping system) in Middle China areas (heading in late August to early September under SD conditions) (Fig. 5b,c). However, those modern *japonica* varieties with weak PS were bred for planting in the mid-season (heading in early to mid-August under LD conditions) in North and North-east China areas (Fig. 5b,c). Because functional *Ghd7* and *DTH8* alleles are also contribute to high grain yield (Xue *et al.*, 2008; Wei *et al.*, 2010; Yan *et al.*, 2011), existing varieties with inappropriately strong PS (having the *HGD*-type) can be improved for weaker PS by CRISPR/Cas9 editing of *Hd1*. Therefore, our findings provide valuable information for breeding of high-yield rice varieties with certain genotypes of these genes and appropriate PS degrees as described earlier.

## Acknowledgements

We thank Prof. Xiangdong Liu, Prof. Jun Fang, Prof. Qingyao Shu, Prof. Shiyong Song, Prof. Dekai Wang and Junmin Wang for providing rice materials, and Prof. Guifu Liu and Dr Zifeng Yang for helping with data analysis. This work was supported by

grants from the National Key Research and Development Program of China (2016YFD0100903), the Major Program of Guangdong Basic and Applied Research (2019B030302006), and the National Natural Science Foundation of China (31871596, 31921004). The authors declare there are no competing interests.

## Author contributions

JG and Y-GL conceived this work, analyzed data and wrote the manuscript; WZ and DR performed most of the experiments; MH, KS, JF, JZ, DX, WX, SL, HZ, RQ, WT, RY, HC, XX and LC performed some of the experiments and data analysis. WZ and DR contributed equally to this work.

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Relationship between day-length and latitude in different months in several locations (cities) of China.

**Fig. S2** Identification of *Hd1* as the factor conferring the non-heading phenotype under sustained long-day conditions in a segregating population.

**Fig. S3** CRISPR/Cas9-mediated targeted mutagenesis of *Hd1*, *Ghd7* and *DTH8* in NHLD plants.

**Fig. S4** Phenotypes of the CRISPR/Cas9-based knockout lines of *Hd1*, *Ghd7* and *DTH8*.

**Fig. S5** Expression levels of *Ehd1*, *Hd3a* and *RFT1* of the eight isogenic lines (58-d-old plants) under ALD and ASD conditions.

**Fig. S6** Comparison of the expression of *Ehd1*, *Hd3a* and *RFT1* in the eight isogenic lines (58 day-old plants) between ASD and ALD.

**Fig. S7** Expression of *Ghd7*, *DTH8* and *Hd1* in the seven isogenic lines (58-d-old plants) under ASD and ALD conditions.

**Fig. S8** Test of the interaction of Hd1 with the *Ghd7*-promoter region.

**Fig. S9** Verification of the interactions among Hd1, DTH8 and Ghd7.

**Fig. S10** Haplotypes (type) of *Hd1*, *Ghd7* and *DTH8* in rice (and *O. rufipogon*), and a summary of haplotypes and PS in several representative cultivated and wild rice lines.

**Table S1** Pearson's correlation coefficient between heading time and gene expression level.

**Table S2** Information of 65 landraces, 61 modern *indica* and *japonica* varieties, and a wild rice accession analyzed in the study.

**Table S3** Primers used in this study.

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