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A Major QTL, *Ghd8*, Plays Pleiotropic Roles in Regulating Grain Productivity, Plant Height, and Heading Date in Rice

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ABSTRACT Rice yield and heading date are two distinct traits controlled by quantitative trait loci (QTLs). The dissection of molecular mechanisms underlying rice yield traits is important for developing high-yielding rice varieties. Here, we report the cloning and characterization of *Ghd8*, a major QTL with pleiotropic effects on grain yield, heading date, and plant height. Two sets of near isogenic line populations were developed for the cloning of *Ghd8*. *Ghd8* was narrowed down to a 20-kb region containing two putative genes, of which one encodes the *OsHAP3* subunit of a CCAAT-box binding protein (HAP complex); this gene was regarded as the *Ghd8* candidate. A complementary test confirmed the identity and pleiotropic effects of the gene; interestingly, the genetic effect of *Ghd8* was dependent on its genetic background. By regulating *Ehd1*, *RFT1*, and *Hd3a*, *Ghd8* delayed flowering under long-day conditions, but promoted flowering under short-day conditions. *Ghd8* up-regulated *MOC1*, a key gene controlling tillering and branching; this increased the number of tillers, primary and secondary branches, thus producing 50% more grains per plant. The ectopic expression of *Ghd8* in *Arabidopsis* caused early flowering by 10 d—a situation similar to the one observed by its homolog *AtHAP3b*, when compared to wild-type under long-day conditions; these findings indicate the conserved function of *Ghd8* and *AtHAP3b* in flowering in *Arabidopsis*. Our results demonstrated the important roles of *Ghd8* in rice yield formation and flowering, as well as its opposite functions in flowering between rice and *Arabidopsis* under long-day conditions.

Key words: Near isogenic lines; CCAAT-box binding protein; ectopic expression; flowering pathway; yield formation.

INTRODUCTION

Rice yield and heading date are two distinct traits that are controlled by quantitative trait loci (QTLs). The two traits are influenced both by the plant's genetic makeup and environmental factors. Many yield-related QTLs/genes have been isolated in rice in recent years. For example, *Moc1* and *IPA1* regulate tillers (Jiao et al., 2010; Li et al., 2003); *GS3*, *GW2*, and *GW5/qSW5* control grain shape and grain weight (Fan et al., 2006; Shomura et al., 2008; Song et al., 2007; Weng et al., 2008), and *Gn1a*, *APO1*, and *DEP1* regulate grain number (Ashikari et al., 2005; Huang et al., 2009; Ikeda et al., 2007). *Ghd7*, which encodes a CCT-domain-containing transcription factor, was reported to regulate rice grain yield, plant height, and heading date. The natural variation of *Ghd7* is an important regulator of heading date and yield potential in rice (Xue et al., 2008).

The adaptation of flowering plants is largely determined by their flowering time, which is mainly controlled by photoperiod and temperature (Izawa, 2007). *Arabidopsis* is a typical long-day (LD) flowering plant whose flowering pathways have been well studied: the key flowering mechanism in *Arabidopsis* is mediated by *CO* (*CONSTANS*), which regulates the production of the florigen (FT) (Corbesier et al., 2007; Kardailsky et al., 1999; Kobayashi et al., 1999; Putterill et al., 1995; Samach et al., 2000). In contrast, rice is a typical short-day (SD) flowering plant whose flowering (heading) is greatly affected by day length. Recently, a series of rice homologs of the genes in the *CO–FT* pathway, namely *Hd1* and *Hd3a*, have been

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identified (Kojima et al., 2002; Yano et al., 2000). The function of *Hd1* is opposite to that of its *Arabidopsis* homolog, *CO*. Under long-day conditions, *Hd1* delays heading in rice, whereas *CO* promotes flowering in *Arabidopsis* (Putterill et al., 1995; Yano et al., 2000). *Ehd1*, a rice-specific flowering regulator, plays a crucial role in the photoperiodic control pathway of rice heading by regulating the expression of the rice florigen genes, *Hd3a* and *RFT1* (Doi et al., 2004; Komiya et al., 2008, 2009). Suppression of *Ehd1* delays heading under SD conditions (Doi et al., 2004). *Ehd1*- and *Hd1*-mediated rice flowering pathways have been regarded as independent (Doi et al., 2004; Izawa, 2007; Komiya et al., 2009). Recently, the heterotrimeric heme activator protein (HAP) complex, composed of the three subunits, HAP2, HAP3, and HAP5, was reported to interact with CO to regulate flowering in *Arabidopsis* (Ben-Naim et al., 2006; Wenkel et al., 2006). The HAP complex binds to the CCAAT box, a *cis*-acting element present in approximately 25% of eukaryotic gene promoters (Bucher, 1990; Maity and de Crombrugghe, 1998; Mantovani, 1999). In yeast and mammals, each subunit of the HAP complex is encoded by a single gene, while in plants, it is encoded by a gene family (Gusmaroli et al., 2001; Thirumurugan et al., 2008). In rice, there are 11 homologous genes (*OsHAP3A–OsHAP3K*) encoding the OsHAP3 unit (Thirumurugan et al., 2008). Four *HAP3* genes were functionally analyzed (Masiero et al., 2002; Miyoshi et al., 2003); the *OsHAP3A*, *OsHAP3B*, and *OsHAP3C* genes are involved in chloroplast biogenesis (Miyoshi et al., 2003).

Undoubtedly, the identification of more rice-yield-related and flowering genes/QTLs will provide us with more opportunities to breed diverse high-yield varieties (Zhang et al., 2008). Understanding the molecular mechanism of such genes could optimize the relationship between yield and heading date. In addition, the functional dissection of more gene-regulated yield traits with different approaches has provided us with the chance to further investigate the molecular mechanisms underlying rice yield (Hu et al., 2008; Luo et al., 2008; Wang et al., 2009). In our previous study (Zhang et al., 2006), we identified a major QTL on chromosome 8, *Ghd8*, which controls grain yield, heading date, and plant height. We report here the cloning and characterization of *Ghd8*. *Ghd8* encodes the OsHAP3 subunit of the HAP complex, and it acts upstream of *Ehd1*, *Hd3a*, and *RFT1*. *Ghd8* coordinates the development of rice panicles and heading.

RESULTS

Fine Mapping of *Ghd8*

In our previous study, the near isogenic line (NIL) of a rice QTL simultaneously controlling plant height, heading date, and the number of grains per panicle on chromosome 8 (referred to as *Ghd8*) was obtained within an F_7 inbred line following a trait-performance-derived NIL strategy; the male parent HR5 allele contributed to delayed heading, tall culms, and large panicles (Zhang et al., 2006). *Ghd8* greatly contributed to rice heading, plant height, and yield-related traits (Figure 1A–1D). The NIL-F_2 (HR5) population of 2256 individuals was investigated for grains per panicle, heading date, and plant height. In total, 326 plants with early heading, a small panicle, and a short culm were selected for *Ghd8* fine mapping. *Ghd8* was narrowed down to a 70-kb region flanked by the markers SEQ3-1 and SEQ5-1 (Figure 2A and Supplemental Table 1). According to the Rice Genome Annotation Project and the RiceGE (Rice Functional Genomic Express) Database, 11 genes were predicted within this region. The gene Loc_08g07740 was predicted to encode a histone-like transcription factor and archaeal histone family protein (CBFD_NF-YB, CCAAT box-binding factor) (http://pfam.janelia.org/search/sequence). In addition, another NIL-F_2(93–11) population containing 17 800 plants was used for fine mapping the QTL and finally narrowed *Ghd8* down to a 20-kb region (Figure 2B). There were two predicted genes in the region containing Loc_08g07740; therefore, Loc_08g07740 was the likely candidate for *Ghd8*.

Complementary Test of *Ghd8*

Comparative sequencing of the gene Loc_08g07740 among the four parental lines Zhenshan 97 (ZS97), 93–11, ZS, and HR5 revealed many nucleotide variations, which caused amino acid changes. HR5 shared the same allele as Nipponbare and had many polymorphisms in both the promoter and the coding region of Loc_08g07740, compared to ZS and ZS97 (both carried nonfunctional alleles). In particular, the single nucleotide polymorphism (SNP) at the position +322 bp from the initiation codon ATG caused a frameshift mutation and led to a premature termination codon in ZS. In ZS97, a deletion of 1116 bp in the 3′ region resulted in a truncated protein that lacked seven amino acids in the C-terminus, compared to 93–11 (Figure 2C). This suggested that Loc_08g07740, which encodes the HAP3 subunit, may be the candidate for *Ghd8*.

To test this prediction, we introduced both *Ghd8* alleles of 93–11 (*Ghd8* (93–11)) and Nipponbare (*Ghd8*(Nip)) by *Agrobacterium*-mediated transformation into NIL^{ZS}, which has a small panicle, short stature, and early heading compared to NIL^{HR5}. The T_1 families of the T_0 transformants containing a single-copy of the *Ghd8* (93–11) and (*Ghd8* (Nip)) alleles were analyzed for their phenotypic effects under natural LD conditions (13–14-h day length). The positive T_1 plants showed significantly more grains, delayed heading and taller culms compared to the transgenic negative plants (Figure 1). The negative *Ghd8* transgenic plants (NIL^{ZS-}) showed a performance similar to their recipient line NIL^{ZS} in heading date, grain yield, grains per panicle, and plant height (Table 1). On average, a positive T1 plant (NIL^{93-11+}/NIL^{Nip+}) resulted in increases above 60% in grain yield per plant and grains per panicle compared to the recipient NIL^{ZS} and the negative T1 plant (NIL^{ZS-}) (Figure 1). The NIL^{Nip+} plant showed a performance similar to that of the NIL carrying the HR5 homozygotes (NIL^{HR5}) in grain yield, grains per panicle, and plant height;

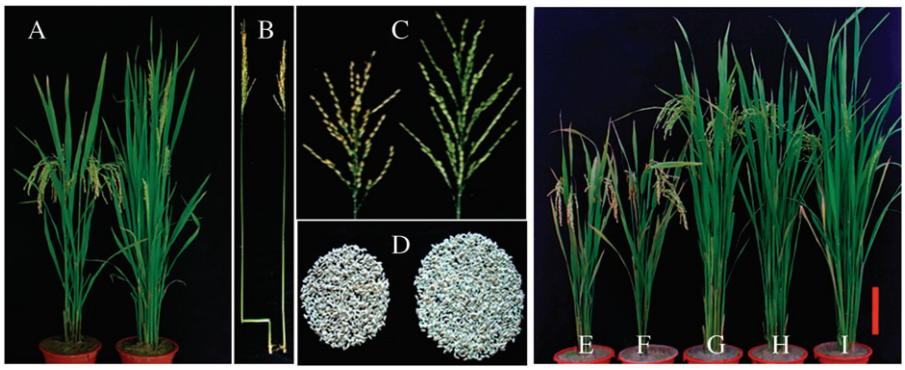

Figure 1. Phenotypes of NILs and Transgenic Rice Transformed with Different Alleles.
(A–D) Phenotype of NILs. Image of whole plants (A), main stems (B), panicles (C), and grain yield per plant (D) for NIL^{ZS} (left) and NIL^{HR5} (right). **(E–I)** Phenotypes of NILs and transgenic rice with different alleles. The plants are NIL^{ZS} (E), NIL^{ZS-} (F), NIL^{HR5} (G), NIL^{Nip+} (H), NIL^{93-11+} (I), respectively. The photograph was taken when NIL^{ZS} reached maturity. Bar = 20 cm.

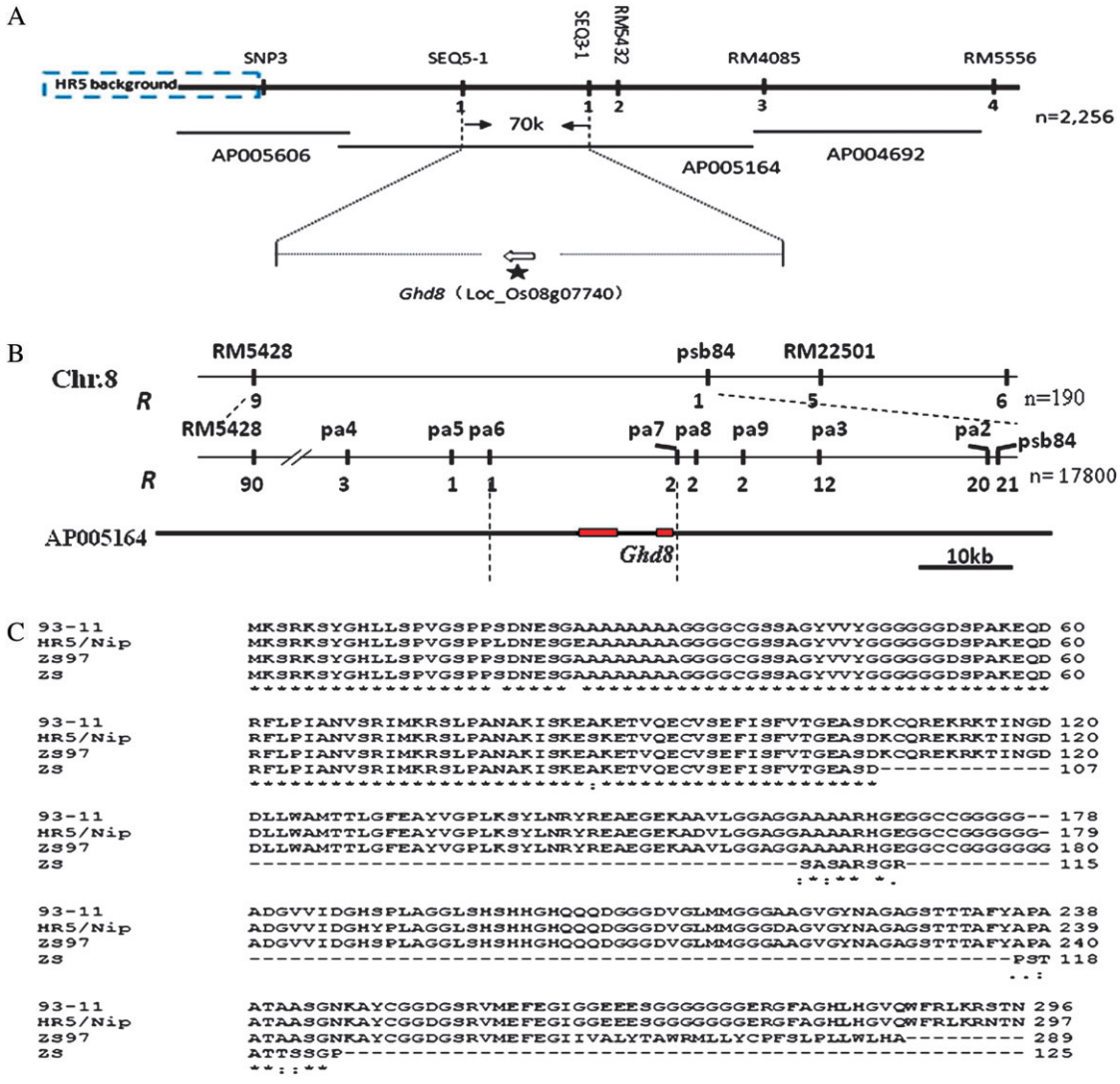

Figure 2. High-Resolution *Ghd8* Fine Mapping and Protein Alignment of the Four Mapping Alleles.
(A) Fine map of *Ghd8* using the NIL-F_2 (HR5) population.
(B) Fine map of *Ghd8* using the NIL-F_2 (93–11) population. The numbers below the markers represent the number of recombinants between the markers and *Ghd8*.
(C) Protein alignment of *Ghd8* from the predicted protein of four parental lines (9311, HR5 or Nipponbare, ZS97 and ZS) using the EMBL software CLUSTAL 2.0.12 multiple sequence alignment tool.

Table 1. Trait Measurements of NILs and Transgenic Plants in the T_1 Generation derived from T_0 Plants with a Single-Copy under Natural Field Conditions in 2009.

Genotype	Plants	HD	PH (cm)	GPP	Tillers	GY (g)
NIL^{ZS}	30	77.1 ± 2.4	78.5 ± 2.1	138.1 ± 27.5	7.3 ± 1.2	15.1 ± 3.4
NIL^{HR5}	30	86.7 ± 1.7	97.8 ± 4.1	223.5 ± 17.9	8.7 ± 1.8	23.8 ± 6.1
p-value		9.7×10^{-26}	2.6×10^{-31}	6.4×10^{-21}	0.0003	3.4×10^{-9}
NIL^{Nip+}	20	86.5 ± 0.99	96.8 ± 5.9	216.3 ± 19.5	8.5 ± 1.2	21.3 ± 5.3
NIL^{Nip-}	20	75.3 ± 0.91	76.9 ± 1.6	131.4 ± 19.4	7.6 ± 1.1	14.8 ± 3.8
p-value		2.1×10^{-14}	6.9×10^{-32}	1.2×10^{-16}	0.01	3.6×10^{-5}
NIL^{93-11-}	11	76.0 ± 2.6	75.7 ± 2.3	123.5 ± 20.3	7.2 ± 1.2	15.4 ± 1.2
NIL^{93-11+}	19	95.8 ± 1.5	97.1 ± 3.7	239.2 ± 17.7	9.9 ± 1.3	26.3 ± 8.0
p-value		6.1×10^{-22}	9.9×10^{-17}	3.8×10^{-16}	2.8×10^{-9}	0.00003

HD, heading date; PH, plant height; GPP, grains per panicle in main culm; GY, grain yield. NIL^{ZS} and NIL^{HR5} mean the near isogenic line carrying homozygous alleles for ZS and HR5, respectively. NIL^{+} and NIL^{-} mean the positive and negative transgenic plants carrying Nipponbare and 93–11 alleles with NIL^{ZS} as the receipt.

however, the transformants with the *Ghd8* (93–11) allele were delayed in flowering by 9 d, thus indicating the strong effect of the 93–11 allele on heading date (Table 1). In summary, the complementary test confirmed that the candidate gene was in fact *Ghd8*.

Characterization of *Ghd8*

The expression of *Ghd8* and other *OsHAP3* genes has been detected in 37 RNA samples of all tissues from ZS97 at different developmental stages using Affymetrix rice gene chips (Wang et al., 2010). *Ghd8* is expressed at a low level in all organs at different developmental stages, like *OsHAP3H* (namely *Ghd8* in this study), whose expression was too low to be detected by RT–PCR in the tissues investigated (Thirumurugan et al., 2008). However, three *OsHAP3* genes were highly expressed in all tested organs at different developmental stages. One gene (Loc_OS02g49410) showed preferential expression in the panicle and endosperm (Supplemental Figure 1). These findings indicated that different members of the *OsHAP3* family have different functions in the rice lifecycle. To check the spatio-temporal expression of *Ghd8* under LD conditions, RNA *in situ* hybridization was performed with roots, young leaves, stems, and young panicles of NIL^{HR5}. *Ghd8* was highly expressed in the phloem of leaves, the vascular system of the stem, and especially in cells of the inflorescence meristem. The inflorescence meristem formed bracts and inflorescence branches (Itoh et al., 2005), which probably determined panicle size (Supplemental Figure 2).

To assay the sub-cellular localization of GHD8, the coding sequence of GHD8 was fused to yellow fluorescent protein (YFP); in contrast, GHD7, which is a nuclear protein, was fused to cyan fluorescent protein (CFP). Both fluorescent proteins were individually driven by the constitutive 35S cauliflower mosaic virus promoter. The constructs were co-transfected into rice protoplasts of etiolated seedlings by polyethylene glycol. GHD8 co-localized to the nucleus with GHD7 (Supplemental Figure 3).

Photoperiod Sensitivity of *Ghd8*

We tested the phenotypes of NIL^{ZS} and NIL^{HR5} under SD (9-h light, 15-h dark) and LD (13.5-h light and 10.5-h dark) conditions. There was no difference in grains per panicle, heading date, and plant height under SD conditions (Table 2). In contrast, NIL^{HR5} was delayed in heading by 7.8 d and was accompanied by an increase of 17.3 cm in plant height and by an average of 81.6 more spikelets per panicle under LD conditions (Table 2). Accordingly, the heading date of the transgenic plant NIL^{ZS+} was delayed by up to 20 d compared to the negative transgenic plant NIL^{ZS-} (Table 1). In contrast, $ZS97^{Nip}$ showed earlier heading by 3.6 d under SD conditions and later heading by 1.5 d under LD conditions, when compared to ZS97. A similar phenotypic variation pattern was also observed in transgenic plants $ZS97^{Nip+}$ (Table 2). Overall, these results showed that *Ghd8* is sensitive to photoperiod.

Ghd8 Action on Heading Date

To understand the role of *Ghd8* in flowering in rice, we compared the expression patterns of *Ghd8* and five other key genes (*Hd1*, *Ehd1*, *OsMADS50*, *Hd3a*, and *RFT1*) in the photoperiodic flowering pathway between NIL^{HR5} and NIL^{ZS} (Doi et al., 2004; Kojima et al., 2002; Komiya et al., 2009; Ryu et al., 2009; Yano et al., 2000). Under SD conditions, the expression patterns of *Ghd8*, *Ehd1*, and *Hd3a* were almost identical between the two NILs (Supplemental Figure 4). This result agreed with the finding that there was no difference in heading date between the two NILs under SD conditions (Table 1). Under LD conditions, the expression levels of *Ehd1*, *Hd3a*, and *RFT1* in NIL^{HR5} were much lower than those in NIL^{ZS}, but the expression levels of *Hd1* and *OsMADS50* were not different between NIL^{ZS} and NIL^{HR5}. This indicated that the expression of *Hd1* and *OsMADS50* is not affected by *Ghd8* (Supplemental Figure 5). When compared to ZS97, $ZS97^{Nip}$ flowered earlier and later under SD and LD conditions, respectively (Table 2). That is to say, the *Ghd8* allele of Nipponbare promoted flowering under SD conditions and inhibited flowering under LD

Table 2. Performance of Near Isogenic Lines for *Ghd8* under Short-Day and Long-Day Conditions in 2010.

	SD (9 h light)			LD (13.5 h light)		
Genotype	HD (d)	PH (cm)	GPP	HD (d)	PH (cm)	GPP
ZS97	85.0 ± 0.4	63.0 ± 0.7	123.0 ± 11.3	90.3 ± 0.33	88.4 ± 1.0	172.8 ± 9.7
$ZS97^{Nip}$	81.6 ± 0.5**	56.8 ± 0.6**	116.8 ± 2.6	91.8 ± 0.40**	83.4 ± 2.7**	209.3 ± 6.5**
NIL^{HR5}	87.4 ± 0.2	70.9 ± 1.5	153.8 ± 5.1	99.9 ± 0.36**	96.0 ± 1.2**	270.1 ± 4.7**
NIL^{ZS}	87.5 ± 0.2	67.6 ± 0.7	156.7 ± 5.8	92.1 ± 0.37	78.7 ± 2.0	188.5 ± 2.7
$ZS97^{Nip+}$	82.4 ± 0.62*	51.9 ± 0.7**	110.6 ± 6.6	91.5 ± 0.65*	85.9 ± 3.2**	214.4 ± 8.3**
$ZS97^{Nip-}$	84.4 ± 0.53	60.8 ± 1.8	116.8 ± 7.1	89.2 ± 0.62	90.5 ± 3.2	159.3 ± 4.5

HD, heading date; PH, plant height; GPP, grains per panicle in main culm; GY, grain yield.*, ** mean the significant difference between the ZS97 back ground near isogenic lines and ZS97, and *Ghd8* transgenic lines $ZS97^{Nip+}$ and $ZS97^{Nip-}$ at the level of $P < 0.05$ and 0.01 by *t*-test. No difference under SD conditions but significant difference ($P < 0.01$) in heading date, plant height, and grains per panicle in main culm under LD conditions between NIL^{HR5} and NIL^{ZS}.

conditions in ZS97. We also compared the diurnal expression of *OsMADS51* (Kim et al., 2008) under SD conditions, that of *OsMADS50* under LD conditions and that of *OsGI*, *Hd1*, *Ehd1*, *Hd3a*, and *RFT1* under both conditions between positive and negative transgenic plants ($ZS97^{Nip+}$ and $ZS97^{Nip-}$). There was no difference in diurnal expression for *OsGI*, *OsMADS51*, *OsMADS50*, and *Hd1*, but a distinct difference was observed for *Ehd1*, *Hd3a* and *RFT1* between $ZS97^{Nip+}$ and $ZS97^{Nip-}$ (Figure 3A and 3B). These results indicated that *Ghd8* acts upstream of *Ehd1*, *Hd3a*, and *RFT1*.

Ghd8 Action on Panicle Development

We investigated the panicle architecture of NIL^{HR5} and NIL^{ZS} grown under natural LD conditions. Significant differences were observed in panicle length and the number of primary and secondary branches (Supplemental Figure 6). These results indicate that *Ghd8* affected panicle size by acting on panicle branching development. To determine whether *Ghd8* functioned in the shoot apex at the vegetative or reproductive stage, we detected its mRNA in the shoot apical meristem (SAM) of young seedlings, the SAM before phase transition, the inflorescence meristem of primary and secondary branch differentiation stages, and the floret meristem, using RNA *in situ* hybridization. *Ghd8* was highly expressed in cells with meristem activity during almost all developmental stages under LD; however, low *Ghd8* expression occurred in SD (Figure 4). To investigate which genes contribute to panicle phenotype, the expression of 10 genes (Supplemental Table 2) associated with panicle architecture was detected in young panicles at the secondary branch initiation stage (length of 1 mm) from the two genotypes of NIL^{ZS-} and NIL^{ZS+} by quantitative PCR. The expression level of *MOC1* was significantly higher in NIL^{ZS+} than in NIL^{ZS-}. Both quantitative PCR and RNA *in situ* hybridization showed that the expression of *MOC1* in the panicle meristem was higher in NIL^{HR5} than in NIL^{ZS} (Figure 5).

Ghd8 Function in *Arabidopsis*

Ghd8 encodes a CCAAT box-binding protein, which is present in animals and plants and which belongs to the HAP3 subfamily. The rice genome contains 12 HAP3 genes (http://drtf.cbi.pku.edu.cn/), while 13 genes encode HAP3 subunits in *Arabidopsis*. An amino acid sequence comparison of these 25 HAP3 genes in rice and *Arabidopsis* showed that *Ghd8* was the homolog of *AtHAP3b* gene (Supplemental Figure 7), which was confirmed to control flowering in *Arabidopsis*. To test the function of *Ghd8* in *Arabidopsis*, we transferred *Ghd8* into the *Arabidopsis thaliana* Columbia ecotype. The transformants flowered about 10 d earlier than the wild-type under LD conditions, but no phenotypic change was observed under SD conditions (Figure 6A and 6B); these findings mirrored those of *AtHap3b* on flowering (Cai et al., 2007; Chen et al., 2007). In other words, flowering was delayed in a null mutant of *HAP3b*, and overexpression of *HAP3b* caused early flowering under LD conditions. No phenotypic variation was observed under SD within both mutant and overexpressing plants. We also compared the expression patterns of the five flowering genes *GI*, *CO*, *HAP3a*, *HAP3b*, and *FT* between transgenic plants and wild-type plants. There was no difference in the expression patterns of *GI*, *CO*, *HAP3a*, and *HAP3b* between the two genotypes, but a distinct increase in *FT* was detected in transgenic plants (Figure 6C); the latter scenario was similar to that caused by *AtHAP3b*. These results indicated that the function of *Ghd8* in flowering is conserved between rice and *Arabidopsis*.

DISCUSSION

Ghd8 Coordinates Panicle Development and Heading Date

The cloned QTLs/genes that control the number of grains per panicle can be classified according to their functions, into the three following groups: genes (*LAX1*, *FZP*, and *MOC1*) that underlie the process of panicle development, genes (*Gn1a*, *APO1*, and *log*) that regulate the rate of spikelet formation, and genes (*Ghd7*) that regulate panicle differentiation (Xing and Zhang, 2010). In the present study, NIL^{HR5} had longer panicles and more primary and secondary branches, which led to more grains per panicle, when compared with NIL^{ZS}; NIL^{HR5} also showed an increase in yield under LD conditions. The *Ghd8*

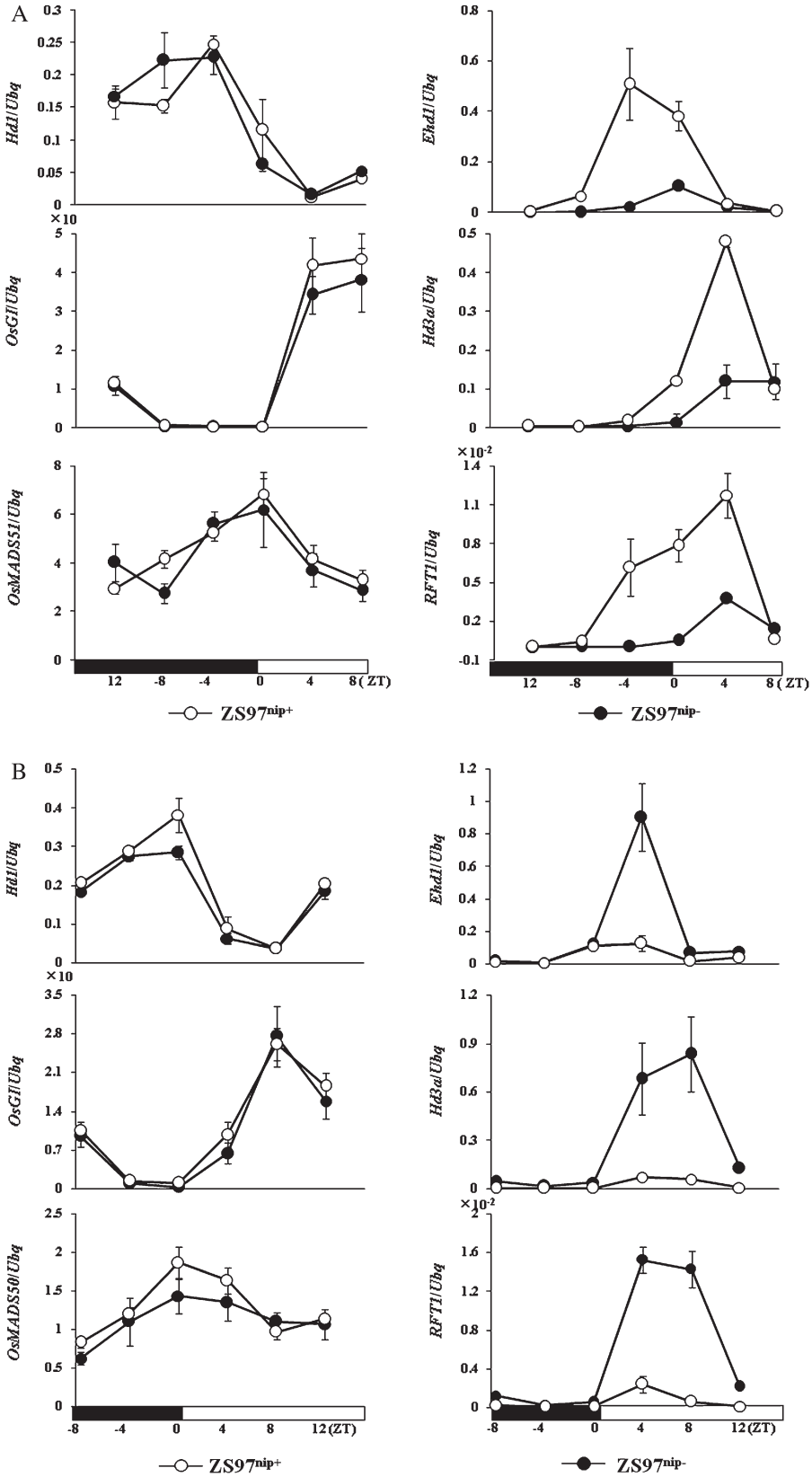

Figure 3. Diurnal Expression of Rice Flowering Genes between the transgenic positive and negative lines.

Diurnal expression of *OsGI*, *Hd1*, *OsMADS51*, *Ehd1*, *Hd3a*, and *RFT1* under SD **(A)** and *OsGI*, *Hd1*, *OsMADS50*, *Ehd1*, *Hd3a*, and *RFT1* under LD **(B)** in ZS97^{nip+} and ZS97^{nip-}, respectively. Each point represents three biological repeats (mean ± standard error (SE)).

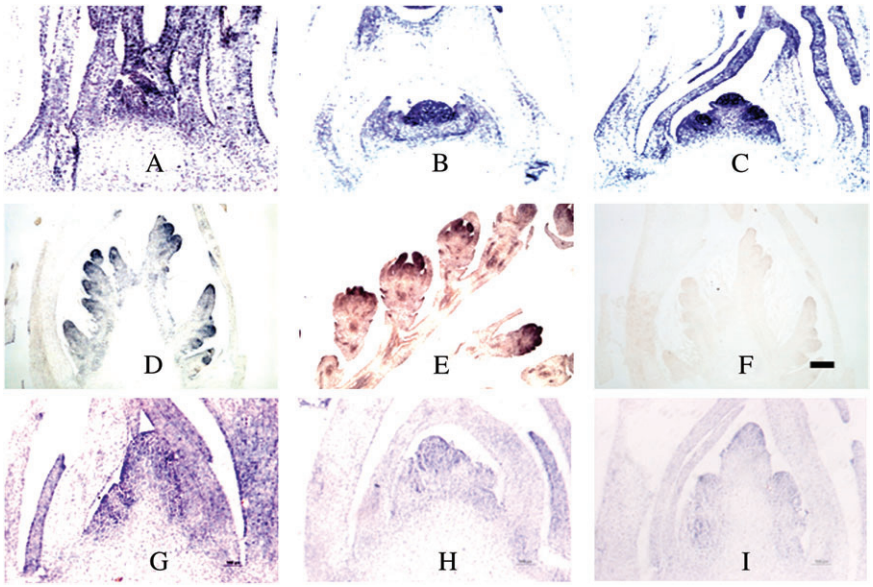

Figure 4. Expression Patterns of *Ghd8* during Panicle Development under LD and SD Conditions.
(A–E) *In situ* hybridization of *Ghd8* in the shoot apical meristem at the vegetative stage (A), shoot apical meristem before phase transition (B), primary branch initiation (C), secondary branch initiation (D), floret meristem (E) under LD conditions and primary branch meristem differentiation (G, H), secondary branch differentiation (I) under SD conditions, and (F) sense probe control; the sample was harvested at 15:00. Bar = 50 μm.

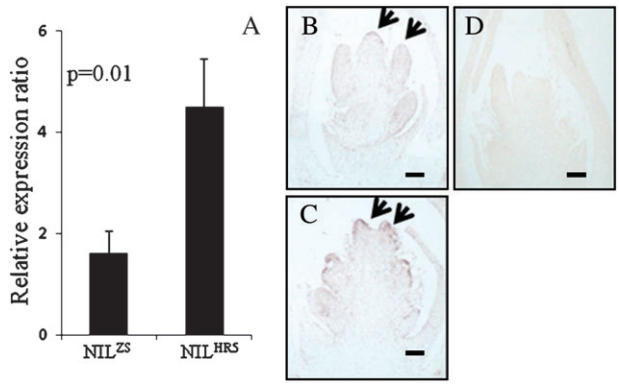

Figure 5. Comparison of *MOC1* Expression Levels during Panicle Development between NIL^{ZS} and NIL^{Hr5}.
(A) Relative expression levels of *MOC1* in NIL^{ZS} and NIL^{Hr5} (samples for RNA extraction were collected from young panicles at secondary branch initiation).
(B–D) *In situ* hybridization of *MOC1* during panicle development in NIL^{ZS} (B) and NIL^{HR5} (C); (D) is the sense probe. Bar = 50 μm.

transgenic plants showed delayed heading, and increased grains per panicle resulted from the increase in primary and secondary branches, when compared to the wild-type. The pleiotropic effects of *Ghd8* were also confirmed in the study of *DTH8* (Wei et al., 2010); *DTH8* is the same gene as *Ghd8*. *Ghd8* was highly expressed in cells with meristematic capacity, perhaps ensuring that more lateral organs develop. *Ghd8* up-regulated *MOC1*, which is involved in tiller branching (Li et al., 2003). Other than its effects on tillers, *MOC1–3* (namely *SPA*) regulated axillary meristem formation during rice panicle development (Oikawa and Kyozuka, 2009). Therefore, *Ghd8* likely functions in branch development during rice panicle development, to produce more lateral panicle organs, and ultimately more spikelets. Thus, *Ghd8* coordinates panicle development and heading date. However, in *Arabidopsis*, the *AtHAP3* family has not been reported to contribute to inflorescence development.

In rice, two independent pathways control flowering; one is regulated by *Hd1* and the other by rice-specific flowering regulator *Ehd1* (Doi et al., 2004; Komiya et al., 2009; Yano et al., 2000). In the present study, the diurnal expression data showed that *Ghd8* was located upstream of *Ehd1*, *Hd3a*, and *RFT1* in the photoperiodic flowering pathway. The genetic interaction between *Hd5* (namely *Ghd8*) and *Hd1* was reported in a QTL study (Lin et al., 2003). Moreover, *Ghd8* up-regulated the expression of *Ehd1*, *Hd3a*, and *RFT1* under short-day conditions, but down-regulated their expression under long-day conditions; this pattern was similar to the regulating rhythm of *Hd1*. Such results may indicate that *Ghd8* links the *Hd1*- and *Ehd1*-dominated pathways to control flowering, consequently

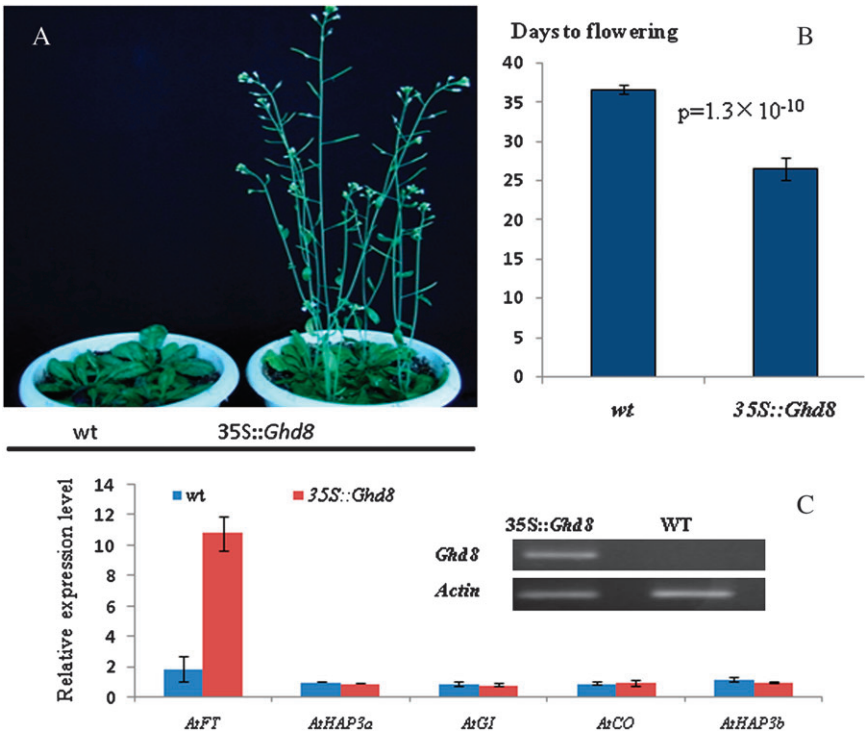

Figure 6. Ectopic Expression of *Ghd8* in *Arabidopsis* and Expression of Related Flowering Genes.
(A) Phenotype of transgenic plant (right) and wild-type (left).
(B) Days to flowering of transgenic plant and wild-type plants.
(C) Comparison of expression for flowering-related genes between transgenic plant and wild-type plant (mean ± SE).

changing the previous notion that *Hd1* acts in parallel with *Ehd1* to control flowering.

Our previously cloned gene, *Ghd7*, has pleiotropic effects similar to those of *Ghd8*. *Ghd7* encodes a CCT domain-containing protein, which is similar to the yeast HEME ACTIVATOR PROTEIN2 (HAP2). *Ghd8* encodes an HAP3 subunit. HAP proteins always form a HAP2/HAP3/HAP5 trimeric complex with DNA binding activity in mammals (Goda et al., 2005; Tuncher et al., 2005). In rice, each HAP subunit is encoded by a gene family; therefore, various possibilities were available to form different HAP complexes, which regulate multi-developmental processes. This explanation may account for the pleiotropic effects of these *Ghd7*/*Ghd8* genes. In addition, the relationship between *Ghd7* and *Ghd8* could be very interesting. However, like Zhenshan 97 (Xue et al., 2008), all the NILs of *Ghd8* in this study were lacking *Ghd7* alleles, because no amplicon was obtained in the NILs with a pair of *Ghd7*-specific primers. NILs carrying functional alleles of both *Ghd7* and *Ghd8* are being developed for the dissection of their genetic relationship.

Effects of *Ghd8* Depend on the Genetic Background

We observed a delay of 6 d in heading in NIL^{HR5} compared to the $ZS97^{Nip}$ under LD conditions. While no difference in flowering was observed under SD conditions between NIL^{HR5} and NIL^{ZS}, $ZS97^{Nip}$ promoted flowering, when compared to ZS97 (Table 2). *Ehd1* was up-regulated in $ZS97^{Nip+}$ compared to $ZS97^{Nip-}$ under SD conditions, whereas there was no difference in *Ehd1* expression between NIL^{HR5} and NIL^{ZS}. However, HR5 and Nipponbare share the same *Ghd8* allele. These results indicated that *Ghd8* has different effects under different genetic backgrounds, and there may be another unknown factor that down-regulates *Ehd1* in the NIL^{ZS} background. Furthermore, the transgenic plants NIL^{93-11+} showed a delay in flowering of 9 d, while it had more grains and tillers than in NIL^{Nip+} transgenic plants, indicating that the different alleles have different effects. HAP proteins always function in the form of the HAP2/HAP3/HAP5 complex (Goda et al., 2005; Tuncher et al., 2005). Consequently, we speculated that the *Ghd8* effects depend on the members of HAP2 and HAP5 forming the complex in the genetic background. Thus, for a given *Ghd8* allele, the constitutions of the HAP complex were determined by other subunits, which resulted in varied functions in different genetic backgrounds. Identification of the interacting subunits would allow breeders to better manipulate the gene for adaptability and high-yielding improvement breeding.

Opposite Function of *Ghd8* in Flowering between Rice and *Arabidopsis*

Flowering was delayed in the mutant *Arabidopsis* gene, but flowering was promoted in plants overexpressing *HAP3b* under LD conditions (Cai et al., 2007). The same phenotype could be observed in the ectopic expression of *Ghd8* in *Arabidopsis*. These results indicate that *Ghd8* and *HAP3b* have conserved functions in *Arabidopsis* flowering.

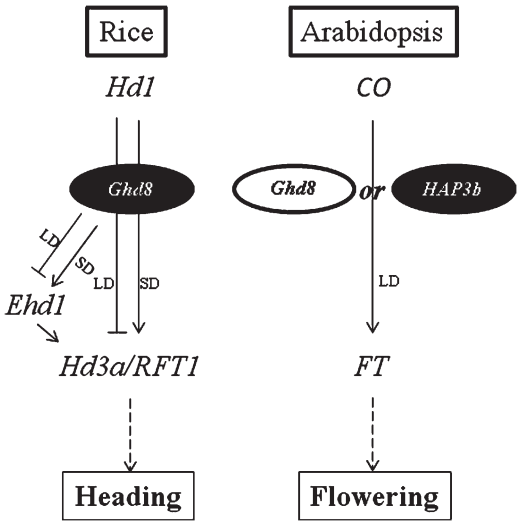

Figure 7. *Ghd8*-Mediated Flowering Pathways Were Conserved in *Arabidopsis* and Rice *CO*/*Hd1* Acts Upstream of *HAP3b*/*Ghd8*, and *HAP3b*/*Ghd8* Regulates *FT*/*Hd3a*/*RFT1*, which Affects Flowering.

Either *Ghd8* or *HAP3b* could promote flowering in *Arabidopsis* under LD conditions. In addition, *Ghd8* could regulate *Ehd1*, which can act on *Hd3a*/*RFT1*, thus affecting flowering in rice.

In *Arabidopsis*, the *CO*-mediated photoperiod flowering pathway, whereby *CO* acts upstream of *FT* and downstream of *GI* to promote flowering under LD conditions, has been well studied (Park et al., 1999; Putterill et al., 1995; Samach et al., 2000). Thus far, no clear result has shown that CO itself can bind DNA directly. It was reported that CO interacts independently with HAP3 and HAP5 (Wenkel et al., 2006). The CCT domain of the CO protein shares amino acid sequence similarities with the DNA-binding domain of the HAP2 protein (Ben-Naim et al., 2006; Wenkel et al., 2006). Overexpression of *AtHAP2* caused late flowering in *Arabidopsis*. Taken together, these results indicated that CO may replace HAP2 to form the CO/HAP3/HAP5 complex to promote flowering (Wenkel et al., 2006). Bioinformatic analysis revealed that *Ghd8* was the closest homolog of *AtHAP3b*, and *Hd1* was the *CO* homolog in rice (Yano et al., 2000). In addition, an epistatic interaction between *Hd1* and *Hd5* (*Ghd8* here) was confirmed (Lin et al., 2003). The complex of GHD8/HD1/HAP5 was expected to be formed to control flowering in rice. In this study, *Ghd8* acted upstream of *Hd3a*/*RFT1*, the rice homolog of *FT*, and the expression of *Hd1* was identical between NILs of *Ghd8*, which may indicate that *Hd1* acts upstream of *Ghd8.* More than that, a similar result was also obtained during the ectopic expression of *Ghd8* in *Arabidopsis*. Thus, we propose that the *HAP3b*/*Ghd8* gene-mediated flowering pathways are conserved between *Arabidopsis* and rice, but they have opposite functions in flowering under LD. Meanwhile, *Ghd8* also acted on the rice-specific gene, *Ehd1*, to regulate flowering in rice (Figure 7).

Overall, the cloning of *Ghd8* has provided a rare opportunity for studying the molecular mechanism of the association between grain yield and heading. Characterization of such mechanisms will lead to a series of discoveries of fundamental importance in plant biology.

METHODS

Plant Material

To clone *Ghd8*, the NIL-F_2 population (NIL-F_2(HR5)) was obtained from a heterozygous F_7 plant derived from a cross between an old version of Zhenshan 97 (ZS) and HR5 (Zhang et al., 2006). The plants in the population that carried HR5 and ZS homozygous alleles were named NIL^{HR5} and NIL^{ZS}, respectively. We also developed an NIL-F_2 population (NIL-F_2(93–11)) from a backcross recombinant inbred plant (BC_1F_8), in which 93–11 was the recipient and new version of Zhenshan 97 (ZS97) was the donor. We developed two NILs that carried *Ghd8* alleles of 93–11 and Nipponbare in the ZS97 background by four or five consecutive backcrosses. They were named $ZS97^{93-11}$ and ZS97 ^{Nip}.

Transformation of *Ghd8* to Rice and *Arabidopsis thaliana*

A clone that contained a candidate of *Ghd8* was selected to test the function of the candidate from the 93–11 BAC library. A 10-kb *Sac*I fragment containing the DNA sequence 3.6 kb upstream the transcription start site, the *Ghd8* ORF, and the 5.7-kb downstream region from a BAC clone were constructed into the binary vector pCAMBIA1301. In addition, a 3.7-kb fragment of Nipponbare containing 1.4 kb of DNA sequence upstream of the initiation codon, the *Ghd8* ORF, and 1.2 kb of the 3′ UTR was constructed into binary vector pCAMBIA1301, respectively. Both fragments contained only one predicted gene, and they were transformed to NIL^{ZS} and ZS97, respectively, by *Agrobacterium*-mediated transformation, using the EHA105 strain. The cauliflower mosaic virus 35S promoter was used to drive the expression of *GHD8* (*HR5*).

The *35S::Ghd8* construct was introduced to *Arabidopsis thaliana* (Columbia ecotype) using the floral dipping method

(Clough and Bent, 1998). Eight transgenic-positive individuals were selected on half MS medium supplemented with 25 μg ml^{-1} of hygromycin. Two transgenic lines, each with a single copy gene insertion, were used for further analysis. The seeds of homozygous transgenic and wild-type plants were sown directly in soil for 10 d under day-neutral conditions (12 h/12 h); then, the seedlings were transferred for the long-day (LD) condition treatment (16-h light/8-h dark photoperiod) and short-day (SD) length treatment (16-h dark/8-h light photoperiod). Flowering time was recorded when the first flower appeared. Leaves were collected for expression analysis. Each leaf sample was composed of five individuals of the same genotype grown under the same conditions.

Sub-Cellular Localization of *Ghd8*

The coding sequence of *Ghd8* (HR5) was fused to PM999–YFP. The fusion protein with the insertion in the right direction was co-transfected into rice protoplasts with *GHD7:CFP* as described by Zhou et al. (2009), with minor modifications. The fluorescence image was obtained using a confocal microscope (Leica, Germany) after incubating the transformed cells in the dark at 28°C for 20 h.

Diurnal Expression Analysis

After growing under neutral day-length conditions for 35 d, the rice plants were transferred to a growth chamber to receive light treatment. The conditions were set as follows: 15 h dark and 9 h light for SD; 9 h dark and 15 h light for LD, with a temperature of 30°C for the light period and 26°C for the dark period. The leaves of the main culm of each plant were harvested every 4 h, for 24 or 48 h. For each time point, leaves from two or three different individuals were collected as biological replicates. RNA was extracted using the TRIzol Reagent (Invitrogen, CA, USA) and treated with DNase I (Invitrogen, CA, USA). cDNA was synthesized from 2 μg of RNA using SuperScript III Reverse Transcriptase (Invitrogen, CA,USA). The quantitative analysis of gene expression was performed with SYBR® Premix Ex Taq™ (TaKaRa, Otsu, JAPAN) on an Applied Biosystems 7500 Real-time PCR System. The data were analyzed with the relative quantification method.

RNA *In Situ* Hybridization

Ghd8 was amplified by the of primer pair, C8dsF and C8dsR (Supplemental Table 1). The product contained 340 bp that were located at the 3′ end of the mRNA; this product was then inserted into the pGEM-T vector (Promega, Madison, USA) for RNA transcription. The respective sense and antisense probes were produced by SP6 and T7 transcriptase labeled with digoxigenin (Roche, Mannheim, Germany). Plant tissues were collected under LD or SD conditions and fixed in FAA solution (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) at 4°C overnight after vacuum pumping. RNA *in situ* hybridization and immunological detection were carried out as described previously by De Block (1993).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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