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A pair of floral regulators sets critical day length for *Hd3a* florigen expression in rice

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The critical day length triggering photoperiodic flowering is set as an acute, accurate threshold in many short-day plants, including rice[1,2]. Here, we show that, unlike the *Arabidopsis* florigen gene *FT*[3], the rice florigen gene *Hd3a* (*Heading date 3a*) is toggled by only a 30-min day-length reduction. *Hd3a* expression is induced by *Ehd1* (*Early heading date 1*) expression when blue light coincides with the morning phase set by *OsGIGANTEA(OsGI)*-dependent circadian clocks. *Ehd1* expression is repressed by both night breaks under short-day conditions and morning light signals under long-day conditions. *Ghd7* (*Grain number, plant height and heading date 7*) was acutely induced when phytochrome signals coincided with a photosensitive phase set differently by distinct photoperiods and this induction repressed *Ehd1* the next morning. Thus, two distinct gating mechanisms—of the floral promoter *Ehd1* and the floral repressor *Ghd7*—could enable manipulation of slight differences in day length to control *Hd3a* transcription with a critical day-length threshold.

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Crop yields are strongly associated with flowering time. In many short-day plants, including rice, the control of flowering has an acute threshold for day length[1,2], although the molecular mechanisms of critical day-length recognition remain unknown. Recent molecular biological advances reveal that *FT* in *Arabidopsis*, a long-day plant, and *Hd3a* in rice encode florigen[4–7], a mobile, long-distance signal that triggers photoperiodic flowering. Molecular genetic studies have revealed how flowering in *Arabidopsis* is accelerated with increasing day length[8–11]. Recently, mathematical modeling from published data has revealed that day length–dependent *FT* expression has no critical threshold in *Arabidopsis*[3].

Identification of natural variations affecting flowering time has revealed genes such as *Ehd1* and *Ghd7*, which encode unique transcriptional regulators in rice[12,13]. *Ehd1*, a B-type response regulator, upregulates *Hd3a* expression and mainly confers short day–dependent flowering promotion in rice[12]. *Ghd7*, encoding a CCT-domain protein homologous to that encoded by wheat *VRN2* (ref. 14), is expressed under long-day conditions and mainly confers long day–dependent flowering repression[13]. Although *Hd1* (*Heading date 1*), a rice ortholog of *Arabidopsis CO* (*CONSTANS*), is another floral promoter under short-day conditions[15], morning *Hd3a* expression is sufficiently conferred by *Ehd1* regardless of *Hd1*[12] (**Supplementary Fig. 1**). Thus, there is likely a unique molecular mechanism for recognizing day length by means of *Ehd1* and *Ghd7* in rice.

Morning expression of *Hd3a*, *Ehd1* and *Ghd7* depends on day length; *Hd3a* and *Ehd1* are expressed only under short-day conditions, whereas *Ghd7* is expressed only under long-day conditions[12,13,16] (**Supplementary Fig. 2**). To reveal whether these genes are regulated by critical day lengths, we examined their expression under various day lengths (**Fig. 1**). In the wild-type (WT) plants, although *Hd3a* mRNA was highly expressed at day lengths of ≤13 h, its expression decreased markedly, to about one-tenth of the high expression, at a day length of 13.5 h and became undetectable at day lengths of ≥14 h (**Fig. 1a**). There was therefore a critical day length of around 13.5 h for *Hd3a* expression, consistent with previous reports that long-day repression of rice flowering becomes evident at day lengths >13 h[1,17,18]. *Ehd1* was expressed at day lengths of ≤13 h and became undetectable at day lengths of ≥13.5 h (**Fig. 1d**), consistent with regulation of *Hd3a* expression by *Ehd1* (ref. 12). *Hd1* deficiency did not clearly affect the critical day-length setting for *Hd3a* expression (**Supplementary Fig. 3**). In the day-length range from 13 h to 13.5 h, *Ehd1* and *Hd3a* expression gradually but significantly decreased in response to every 10 min day-length increase, revealing the time resolution of critical day length in rice (**Supplementary Fig. 4**). In contrast, *Ghd7* mRNA levels increased with increasing day length and had no critical–day length recognition (**Fig. 1g** and **Supplementary Fig. 4**). Substantial repression of *RFT1* (ref. 19), an *Hd3a* paralog, was observed at day lengths ≥13 h (**Supplementary Fig. 5**); the critical day length setting for *RFT1*was not as acute as that for *Hd3a*.

To characterize the molecular mechanism of this critical-day-length recognition, we studied known photoperiodic-flowering mutants of rice. *osgi* (or the *osgi-1* allele), a mutant defective in *OsGI* (the sole rice ortholog of *Arabidopsis GIGANTEA (GI),* a circadian clock–related gene) with late flowering only under short-day conditions (T.I., M. Mihara, Y. Suzuki, H.I., A. Nagano *et al.*, unpublished data), exhibited low levels of *Hd3a*, *Ehd1* and *Ghd7* mRNA and day-length insensitivity (**Fig. 1a**,**d**,**g**). Therefore, *OsGI* is required to set critical day length for *Hd3a* through regulation of *Ehd1* and *Ghd7* transcription. In contrast, de-repression of *Ehd1* and *Hd3a* mRNA, along with day-length

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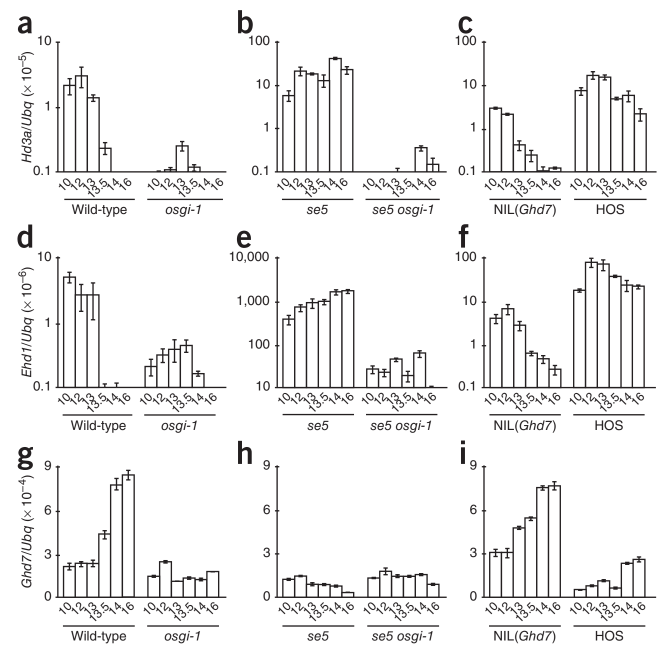

Figure 1 Levels of *Hd3a*, *Ehd1* and *Ghd7* mRNA under various day-length conditions. Relative mRNA levels determined by quantitative RT-PCR. (**a**–**i**) Shown are results for *Hd3a* (**a**–**c**), *Ehd1* (**d**–**f**) and *Ghd7* (**g**–**i**). Wild-type rice plants and mutants are indicated at the bottom of each panel. HOS is a *Ghd7*-deficient cultivar, and NIL(*Ghd7*) is its nearly isogenic line harboring a functional *Ghd7* allele. Numbers of hours of light in 24 h light-dark cycles are indicated on the *x* axis. After entrainment of plants by different day-length conditions for 5 d, samples were collected 3 h after dawn. In **a**–**f**, the *y* axis is a log scale. In **a**–**i**, the average values and standard deviation (s.d.) from three PCR measurements are shown. Results are representative of three independent experiments.

insensitivity, were observed in *se5* plants[20–22] (**Fig. 1b**,**e**), phytochrome-deficient mutants that exhibit day length–insensitive early flowering. Loss of *Ghd7* expression in *se5* plants suggested that phytochromes mediate light signals to induce *Ghd7* (**Fig. 1g**,**h**). De-repression of *Ehd1* and *Hd3a* was not observed in *se5 osgi-1* double mutants (**Fig. 1b**,**e**), indicating that *OsGI* is required for induction of *Ehd1* and *Hd3a,* but that it does not work through *Se5* and *Ghd7* repression.

Next, we examined the role of *Ghd7* in *Ehd1* and *Hd3a* expression. We introgressed a functional allele of *Ghd7* from plants of the Kasalath line, an *aus* cultivar, into plants of the Hoshinoyume (HOS) line, a *Ghd7*-deficient japonica cultivar, to produce a nearly isogenic line carrying a functional *Ghd7* allele, termed NIL(*Ghd7*) (**Supplementary Fig. 6**). The *aus* subgroup of rice cultivars is close to the *indica* subspecies but is distinct from the *japonica* subspecies. As in the WT plants (**Fig. 1a**,**d**), *Ehd1* and *Hd3a* mRNAs were strongly repressed in day lengths >13.5 h in NIL(*Ghd7*) plants (**Fig. 1c**,**f**). In HOS plants, de-repression of both *Ehd1* and *Hd3a* was observed at all day lengths (**Fig. 1c**,**f**), implying that *Ghd7* represses *Ehd1*, which results in *Hd3a* repression. The less acute long-day repression of *Ehd1* and *Hd3a* observed in NIL(*Ghd7*) plants may be due to differences in genetic background.

Morning *Ehd1* induction under short-day conditions (**Supplementary Fig. 2**) led us to investigate light-quality effects on *Ehd1* induction by using *se5* mutants, as *Ehd1* and *Hd3a* repression by *Ghd7* through phytochromes was lost in these plants (**Fig. 1b**,**e**). In *se5* plants under blue-light short-day conditions, but not red-light conditions, transient and strong *Ehd1* expression was observed 2 h after dawn (**Fig. 2a**). *Ehd1* induction was not detected in continuous dark conditions. Because morning expression of *Ehd1* was not observed in *se5 osgi-1* mutants (**Fig. 2a**), we monitored the response of *Ehd1* induction to blue light pulses in *se5* plants at various circadian phases after entrainment under short-day or long-day conditions (**Fig. 2b**). *Ehd1* expression resulted in clear gating responses to blue light pulses, with a peak expression between 10 and 12 h after dusk, regardless of the entrained day-length conditions. In *se5 osgi-1* mutants, however, no *Ehd1* induction by blue light pulses was consistently observed. Thus, *OsGI* shapes a 'gate' around dawn for blue light signals in *Ehd1* expression regardless of photoperiod (where 'gate' here means a sensitive phase set by circadian clocks in response to a given stimuli, such as light). Our finding that the level of OsGI protein was lowest at about dawn (**Supplementary Fig. 7**) suggests that OsGI does not directly control the morning gate for *Ehd1* induction but rather controls expression of downstream gate component(s).

Brief exposure to red light at midnight—the so-called 'night break'—inhibits flowering of rice under short-day conditions[23]. Therefore, we next examined whether blue-light morning induction of *Ehd1* was affected by night breaks. Using 10-min red light pulses at various times in WT plants entrained under short-day conditions, we measured induction of *Ehd1* and *Hd3a* mRNAs the morning after the pulses (**Fig. 3a**). *Ehd1* and *Hd3a* expression were suppressed maximally by night breaks at approximately midnight. To clarify how red light suppressed gated *Ehd1* induction, we examined *Ehd1* induction in WT plants by blue light pulses after red light–pulse treatment at midnight (**Fig. 3b**). As in *se5* plants (**Fig. 2b**), single blue light pulses to WT plants under short-day conditions induced *Ehd1* expression, which peaked 12 h after dusk (**Fig. 3b** and **Supplementary Fig. 8**). Red light pulsing 6 h after dusk (the time of most effective suppression of *Ehd1* morning induction; **Fig. 3a**) severely suppressed *Ehd1* induction by subsequent blue light pulses 9 h and 12 h after dusk (**Fig. 3b**). Only

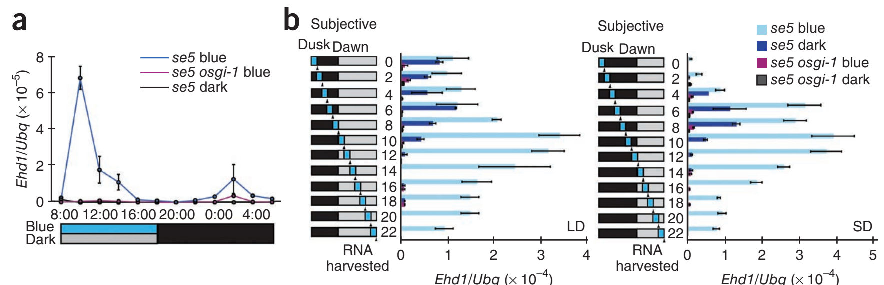

Figure 2 Analysis of *Ehd1* expression. (**a**) Diurnal expression patterns of *Ehd1* in *se5* and *se5 osgi-1* rice plants grown under short-day conditions (10 h light, 14 h dark) in blue light. The blue bar represents the blue-light period and the black bar represents the period of darkness. The gray bar represents dark treatment instead of blue light. (**b**) Gated expression of *Ehd1* expression responsive to blue light. *se5* and *se5 osgi-1* plants were entrained under long-day (left) or short-day conditions (right) for 14 d. Plants were transferred to continuous dark at dusk (0). Replicate samples were then each exposed once to 2 h of blue light at times differing by 2 h. Black and gray bars in panels to left of graphs represent subjective night and day. The blue bands in the panels represent the 2 h blue-light exposure periods. Arrowheads represent the timing of harvest for RNA extraction. Average values and s.d. from three RT-PCR data are shown. All data are representative of two independent experiments. LD, long-day conditions; SD, short-day conditions.

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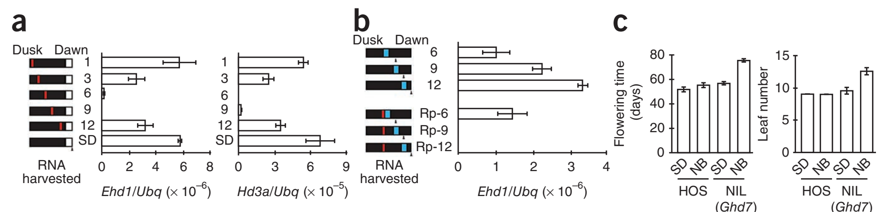

Figure 3 Analysis of the effect of night breaks. (**a**) Night breaks of red light pulses suppress *Ehd1* and *Hd3a* expression the next morning. A 10-min red light pulse was given at different times from dusk, as indicated at left (*y* axes on the two graphs represent hours after dusk) to wild-type rice plants entrained under short-day conditions. Samples were then collected 3 h after dawn. (**b**) Red light pulses suppress blue light–dependent expression of *Ehd1*. Red light pulses (Rp) and/or blue light pulses were given as indicated. In both **a** and **b**, wild-type plants were entrained under short-day conditions for 14 d. Black and white bars in left panels represent dark and light periods. Red or blue bands are 10-min red light pulses or 1-h blue light pulses. Arrowheads represent timing of harvest for RNA extraction. Average values and s.d. from three RT-PCR data are shown. All data are representative of two independent experiments. (**c**) Effects of night break on flowering in HOS and NIL(*Ghd7*) plants. Flowering time was determined by counting the days to flowering after sowing (left) and/or the number of leaves (right) at the onset of flowering. A 10-min white light was applied at midnight for 20 d starting at 3 weeks after germination for the night break. Average values and s.d. are shown ($n = 7$). SD, short-day conditions; NB, night break.

sequential exposure to blue light just after the red light pulse was able to induce *Ehd1*, indicating the need for a time lag after red-light pulses for effective red light suppression of blue light induction of *Ehd1*.

Consistent with its being under phytochrome control (**Fig. 1g**,**h**), *Ghd7* was induced by red light pulses in WT plants under long-day conditions (**Supplementary Fig. 9**). The blue light pulses required for *Ehd1* induction were much longer than the red light pulses needed for *Ghd7* induction (**Supplementary Figs. 9** and **10**). Whereas night breaks at midnight delayed flowering in NIL(*Ghd7*) plants under short-day conditions, the *ghd7*-deficient cultivar HOS was insensitive to night breaks (**Fig. 3c**); night breaks under short-day conditions thus suppressed flowering through phytochrome-mediated induction of *Ghd7*. We thus monitored *Ghd7* induction by using red light pulses in various circadian phases in WT plants entrained under either long-day or short-day conditions (**Fig. 4**). Under long-day conditions, *Ghd7* expression was gated with a photo-inducible phase pattern clearly peaking at subjective dawn (08:00) (**Fig. 4**). Under short-day conditions, however, the peak photo-inducibility of *Ghd7* shifted to 10 h before subjective dawn (22:00) (**Fig. 4**). Thus, *Ghd7* is regulated by gated phytochrome signaling. Unlike the *Ehd1* gate, the *Ghd7* gate opened differently depending on day length. Under short-day conditions, we reproducibly observed a small bump 6 h after the first peak (04:00) (**Fig. 4**). *osgi-1* plants exhibited reduced *Ghd7* mRNA at the first peak but no substantial reduction at the second smaller peak (06:00) (**Supplementary Fig. 11**). This suggests that *OsGI* is at least required for the first peak production of the *Ghd7* gate, in contrast to the obligate requirement of *OsGI* for the *Ehd1* gate (**Fig. 2b**).

Using a red light pulse just before a blue light pulse, we found that *Ghd7* could not repress blue-light induction of *Ehd1* (**Fig. 3b**), indicating that *Ghd7* needs a time lag between red and blue light pulses to exert its repressive activity after transcriptional induction. Therefore, morning *Ghd7* transcription under long-day conditions may be required to repress blue-light induction of *Ehd1* the next morning. We first addressed this question using a physiology-based approach of modifying photoperiods. This initial experiment failed, possibly because of the quick damping of rhythmic expression of circadian clock–related genes under darkness in rice (**Supplementary Fig. 12** and **Supplementary Note**). We next developed an inducible system for *Ghd7* expression using *HSP*, which encodes a rice heat-shock promoter. We selected the 5′ flanking region of *Oshsp16.9C*, the expression of which is induced by heat but no other stresses[24]. We used this to construct a fusion gene, *HSP::Ghd7*, which we introduced into Kita-ake, another *Ghd7*-deficient rice cultivar. Unlike the control, *HSP::luc* (comprising *HSP* fused to the gene encoding firefly luciferase), some *HSP::Ghd7* transgenic plants exhibited delayed flowering due to moderate reduction of *Ehd1* mRNA even without heat-shock treatment, implying that the *HSP::Ghd7* transgenes were functional (**Fig. 5a** and data not shown). In these transgenic plants, application of a 15-min morning heat-shock 1 h after subjective dawn strongly repressed blue light–induced *Ehd1* the next morning (**Fig. 5a**). Successive adjustment of *Ghd7* induction by changing the duration of heat-shock treatment showed a strong correlation between suppressed morning levels of *Ehd1* mRNA and heat-induced levels of *Ghd7* mRNA on the previous morning (**Fig. 5b**). Thus, the durability of *Ghd7* repression of *Ehd1* depends on *Ghd7* expression levels on the previous morning (**Supplementary Note**).

We showed here that short day–induced expression of *Hd3a* has a critical day length[1,2]. To our knowledge, this is the first report clearly showing a strong link between florigen transcriptional regulation and critical day-length recognition. We further demonstrated that critical day length for *Hd3a* occurs by the interaction of two gating mechanisms (**Supplementary Fig. 13**). One is gating of *Ehd1*, an *Hd3a* activator, by blue light. The other is phytochrome-mediated gating of *Ghd7*, an *Hd3a* repressor. We showed that *Ghd7* strongly represses *Ehd1*. Without *Ghd7* function, *Ehd1* is induced regardless of day-length conditions by means of the coincidence of blue light with a photo-inducible phase set by *OsGI* (**Fig. 2b**). *Ghd7* is

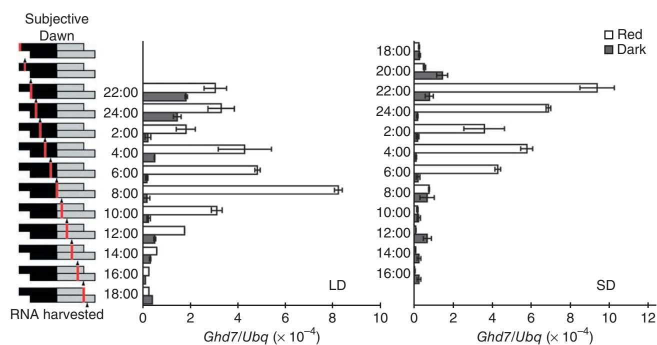

Figure 4 Analysis of gated expression of *Ghd7*. Wild-type plants were entrained under long-day or short-day conditions for 14 d. Plants were transferred to darkness at dusk (22:00 for long day and 18:00 for short day). Replicate samples were then exposed once to a 10 min red light pulse at times differing by 2 h. Acute response of *Ghd7* expression was analyzed 2 h after the beginning of exposure. Red light pulses given at different times are indicated at left; subjective dawn was at 08:00. Black and gray bars in left panel represent subjective night and day. The red bands represent 10 min of red light. Arrowheads represent the timing of harvest for RNA extraction. Average values and s.d. from three RT-PCR data are shown. Data are representative of two independent experiments. LD, long-day conditions; SD, short-day conditions.

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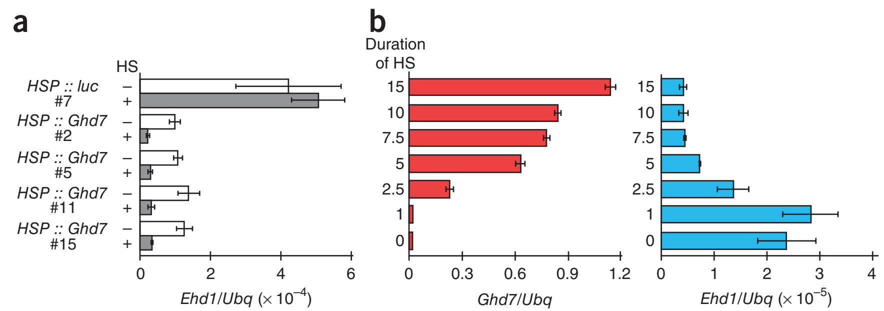

Figure 5 Sustainability of *Ghd7* activity in repressing *Ehd1* expression the next morning. (**a**) T_0 lines of *HSP::Ghd7* and *HSP::luc* were entrained under long-day conditions. Plus and minus indicate with and without heat-shock treatment (42 °C for 15 min), respectively. (**b**) Levels of *Ghd7* expression in the morning determine the de-repression status of *Ehd1* expression the next morning. Lengths (min) of heat-shock pulses are indicated at left of panels. *HSP::Ghd7* line 15 (from **a**) was used for this experiment. Levels of *Ghd7* mRNA (red bars) were measured 2 h after heat-shock treatment. HS, heat shock. (**a**,**b**) Heat-shock treatment was given 1 h after dawn. *Ehd1* expression (blue bars) induced by blue light the next morning was examined 2 h after dawn. Average values and s.d. from three RT-PCR data are shown. Data are representative of two independent experiments.

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also regulated by coincidence of phytochrome signals and another photo-inducible phase, in that peak *Ghd7* inducibility depends on day length. Therefore, morning *Ghd7* expression was decreased by day-length shortening, as peak *Ghd7* inducibility shifted from dawn in long-day conditions to midnight in short-day consitions (**Figs. 1g** and **4**). By these two external coincidence systems, *Ghd7* and *Ehd1* can be triggered by morning light under long-day conditions. Because *Ghd7* induction in the morning can repress *Ehd1* the next morning, critical day-length recognition for *Hd3a* is achieved by release of gated morning *Ehd1* induction only when *Ghd7* mRNA levels during the previous morning are too low to sustain *Ghd7* repressor activity at the next dawn.

Arabidopsis often grows at high latitudes, using floral repression released by vernalization to endure the long winter and flower in the warming spring[25,26]; a single but complex external coincidence system enables *CO* to activate *FT* only under long-day conditions[9–11]. In contrast, typical rice plants germinate in spring, growing vegetatively in summer as long as possible to maximize fitness. The transcriptional regulation of florigen genes conferred by the two gating mechanisms on the floral promoter and repressor could enable the use of slight differences in day length to control flowering time in rice and thus enable them to be grown over a greater climatic range, which would result in more global yields of rice.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. The sequences of *Hd3a*, *Ehd1* and *Ghd7* have been deposited in the NCBI nucleotide database under accession codes AB052942, AB092506 and EU286801, respectively.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

H.I. performed the majority of the experiments. Y.N. and M.Y. provided the NIL(*Ghd7*) plants. T.I. came up with the study design and organized this work. Both H.I. and T.I. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Plant growth conditions. The subspecies japonica rice (*Oryza sativa*) 'Norin 8' was used as the wild type. Seeds were imbibed in darkness (48 h at 30 °C) and then sown in soil. Plants were grown in a growth chamber at 70% humidity under short-day (or long-day) conditions with daily cycles of 10 h (or 14.5 h) of light at 28 °C and 14 h (or 9.5 h) of dark at 24 °C. Light was provided by a metal halide lamp (photosynthetic photon flux density of 450 μmol m^{-2} s^{-1}). For different light regimes, chambers equipped with light-emitting diodes (Sanyo) were used. For blue light, the intensity was 175 μmol m^{-2} s^{-1}; for red light, the intensity was 12.5 μmol m^{-2} s^{-1}. Flowering time was defined as the time when the first panicle emerged.

Production of NIL(*Ghd7*) plants. The Japanese cultivar HOS, defective in *Ghd7*, was crossed with the indica cultivar Kasalath, which harbors a functional *Ghd7* allele. The NIL(*Ghd7*) line was developed by repeated backcrossing with HOS. Introgression of the genomic region of *Ghd7* was followed by genotyping of four markers: RM6338 (41.7 cM) and E0407A (46.4 cM) on the north side and S20922 (50 cM) and C30372 (65 cM) on the south side (**Supplementary Fig. 6**). The region of *Ghd7* in HOS used here was the same as that of the *Ghd7-0a* allele reported previously[12].

Analysis of gene expression. Total RNA was extracted from 14-d-old seedlings by using an RNeasy Plant Mini Kit (Qiagen) in accordance with the manufacturer's instructions. cDNA was synthesized from 5 μg of total RNA by using Superscript II reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed by the Taq-Man PCR method on an ABI PRISM 7900 Sequence Detection System in accordance with the manufacturer's instructions. Gene-specific primers used in this study are listed in **Supplementary Table 1**.

Plasmid construction. The 5′ flanking region of *Oshsp16.9C*, a rice ortholog of *Gmhsp17.3-B*, and *Ghd7* cDNA were amplified by PCR using the primers listed in **Supplementary Table 1**. The resulting PCR products were cloned into the pCR BluntII-TOPO vector (Invitrogen). These clones were sequenced to confirm that there was no nucleotide substitution. The *HSP* promoter fragment digested with HindIII and BamHI to obtain *HSP::luc* was ligated into the HindIII and BglII sites of the entry vector containing *luc* (encoding firefly luciferase) and the *NOS* terminator. The entry vector possessed KpnI at the beginning of *luc* and SacI behind the *luc* stop codon. *Ghd7* cDNA was digested with EcoRI and blunted and then redigested with KpnI. The fragment was replaced with *luc* by ligation at the blunted SacI and KpnI sites. Finally, both entry clones were used in the LR reaction with the destination vector pGWB501 to create a binary plasmid containing *HSP::Ghd7-NOS* and *HSP::luc-NOS* [27]. The LR reaction was performed as described in the manufacturer's manual (Invitrogen).

Plant transformation. A binary vector harboring *HSP::Ghd7* or *HSP::luc* was introduced into *Agrobacterium tumefaciens* strain EHA101 by electroporation. Plasmids were transformed into Kita-ake as described[28,29]. Transgenic plants were selected on medium containing 50 mg/l hygromycin. Hygromycin-resistant plants were transplanted into soil and grown as described above.

Immunoblot analysis. Seedlings were homogenized in 3 vol (1.5 ml) of homogenization buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.1% Tween 20) with a Complete Protease Inhibitor Cocktail (Roche). Extracts were mixed with SDS sample buffer and boiled for 5 min. Samples were then subjected to SDS-PAGE (7.5% polyacrylamide) and transferred to a nitrocellulose membrane (Amersham) by semi-dry blotting. The blot was incubated with α-HA antibody (COVANCE) and then with mouse IgG horseradish peroxidase-conjugated secondary antibody. Detection of the peroxidase activity was performed according to the instruction manual (Pierce).

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