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DELAYED HEADING DATE3, Encoding a Heat Shock Transcription Factor, Delays Flowering Time and Improves Yield in Rice (*Oryza sativa* L.)

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Abstract: Heading date is an essential agronomic trait that affects adaptability and yield in rice (*Oryza sativa*). HSFs (heat shock transcription factors) are a type of transcription factor that responds to environmental stress in organisms. The relationship between the heading date and HSFs has been seldom reported so far. Here, we identified a new heat shock transcription factor, named *DELAYED HEADING DATE3* (DHD3), which can significantly delay the heading date by about 14 days and provide improvements of about 77% potential yield in rice. DHD3 protein is localized in the nucleus and has weak transactivation activity. DHD3 delays the heading date by significantly suppressing *Hd3a* and *RFT1* expression under long-day (LD) and short-day (SD) conditions. Furthermore, the low-temperature condition greatly enhances the delay effect of DHD3 on the heading date (from 16.1% to more than 89.3%). We propose that DHD3 may involve the temperature-regulated signaling pathway of flowering time in rice and has the potential to improve crop yield.

Keywords: flowering time; grain yield; heading date; heat shock transcription factor; low temperature; *Oryza sativa*; rice

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1. Introduction

Rice is an important food crop in the world. Heading date is an important agronomic trait that affects rice yield [1]. There are two florigen genes in rice, *Hd3a* and *RFT1*, which play important roles in regulating the flowering time of rice under short-day (SD) and long-day (LD) conditions, respectively [2]. Rice is a typical short-day plant. Under SD conditions, the initiation of rice flowering is mainly controlled by the GI–Hd1–Hd3a (*GIGANTEA–Heading date 1–Heading date 3a*) pathway [3]. This pathway is similar to the flowering signaling pathway in *Arabidopsis* [4]. OsGI can activate the expression of *Hd1* and promote the early heading date of rice under SD conditions [3]. However, under LD conditions, the expression of *Hd1* inhibited the expression of *Hd3a* and delayed the flowering time of rice [5]. The opposite effects of *Hd1* under SD and LD conditions may be regulated by multiple genes such as *PHYB* (*Phytochrome B*), *Hd6* (*Heading date-6*), *DTH8* (*Days To Heading on Chromosome 8*), and *Ghd7* (*Grain number, plant height, and heading date 7*) [6–10]. In addition, the initiation of rice flowering can also be regulated by another flowering pathway, *Ehd1–Hd3a/RFT1* (*Early heading date 1–hd3a/RICE FLOWERING LOCUS T 1*), which is unique to rice [11]. Ehd1, a B-type response factor, is a unique heading date regulation protein in rice and promotes flowering [12]. Early heading date 2 (Ehd2) [13],

Early heading date 3 (Ehd3) [14], Early heading date 4 (Ehd4) [15], SDG724 (SET domain group protein 724) [16], Ghd7 [17], CONSTANS-Like 4 (OsCOL4) [18], OsLFL1 (O. sativa LEC2 and FUSCA3 Like 1) [19], OsRE1 [20], and other proteins regulate the heading date of rice by controlling the expression of *Ehd1*. Environmental factors, such as low temperature, drought, and gibberellic acid (GA), can also affect the heading date of rice through *Ehd1* [21–23].

Heat shock proteins (HSPs) are molecular chaperones that express ubiquitously in all organisms that maintain or restore protein homeostasis under stressful conditions [24,25]. Heat shock transcription factors (HSFs) are a type of transcription factor that controls the gene expression of HSPs by binding to the *heat shock elements* (*HSEs*) of the gene promotor sites of HSPs [26]. Unlike animals that generally encode only a few HSFs, most plants have more than 20 HSFs, which are essential for plant adaptation to various stressful environments [26]. Based on the sequence homology and conserved domains of these HSFs, HSFs in plants can be divided into three classes: class A, B, and C. The conserved domains of HSF proteins include DNA binding domain (DBD), oligomerization domain (OD), nuclear localization signal (NLS), nuclear export signal (NES), activator motifs (aromatic and large hydrophobic amino acid residues embedded in an acidic surrounding, AHA motifs) [27], and repressor domain (RD). Among them, AHA and RD are the unique structure domains of class A and class B, respectively [28]. Many previous studies reported the functions of HSFs in plants, most of which are related to abiotic stresses such as heat, drought, salt, and cold [29]. The first reported HSF genes in plants were three *HSF* genes in tomatoes, which were induced by heat stress [30]. Liu et al. found that the *Arabidopsis HSFA1* quadruple knockout mutant (*hsfa1a*, *1b*, *1c*, *1d* mutant) had impaired thermotolerance and reduced the expression of most heat-induced genes [31]. *HSFA4A*, *HSFA6B*, *HSFA8*, and *HSFC1* genes are induced by low temperature [32]. Under low temperatures, HSFA1 cooperates with NPR1 to promote the expression of *HSFA1*-related genes and cold acclimation [33]. In addition to responding to extreme temperatures, HSFs are also critical in responding to drought, salinity, osmotic, oxidative stress, and pathogen defense [29].

In rice, *spl7* mutants, allelic mutants of *OsHSFA4D*, show disease mimic spotted leaf phenotype under high temperature [34]. The overexpression of *OsHSFA2E* increases the high-temperature and high-salinity stress tolerance in transgenic *Arabidopsis* [35]. Decreased *OsHSFA4A* expression impairs tolerance to Cadmium in rice [36]. Recently, Zhu et al. reported that the overexpression of *OsHSFA3* in *Arabidopsis* can improve drought tolerance by reducing water loss and reactive oxygen species (ROS) levels [37]. Overexpressing *OsHSFB4D* in rice exhibited enhanced resistance to *Xanthomonas Oryzae* by increasing the expression of *OsHSP18.0-CI* [38]. So far, no HSF has been reported to be related to the heading date in rice.

So far, most reports related to the heading date in rice are focused on the photoperiod pathway. The effect of environmental factors, especially temperature, on rice flowering has been less reported. The mechanism of temperature affecting rice flowering needs more exploration. Here, we identified an HSF associated with rice heading date and named it DELAYED HEADING DATE 3 (DHD3). When *DHD3* was overexpressed in rice, the transgenic plants delayed heading and increased yield under both LD and SD conditions. DHD3 protein is located in the nucleus and has weak transcription activation activity. DHD3 inhibits rice flowering by inhibiting the gene expression of *Hd3a* and *RFT1* under both LD and SD conditions. The inhibitory effect of DHD3 was significantly enhanced when the temperature of the growth environment was lower, suggesting that DHD3 may be involved in temperature regulation of flowering. In brief, DHD3 is a novel transcription factor controlling rice heading and has the potential to increase crop yield.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Oryza sativa L. ssp. *japonica* cv. Nipponbare and Kitaake were used for the transformation of rice in this study. All plants were grown in paddy fields in Beijing (116°13′ E,

40°13′ N) during summer as the natural long-day (NLD) condition. Plants were also planted in artificial light incubators under a relative humidity of 70%. The controlled long-day (LD) condition is 14 h light, 30 °C/10 h dark, and 25 °C, and the controlled short day (SD) condition is 10 h light, 30 °C/14 h dark, and 25 °C. To investigate the flowering time in low temperature treatment, plants were grown at 20 °C in SD conditions. In 2017, the *ehd1*, *ehd2*, *ehd4*, *hd1*, *ghd7*, *dth8*, *osprr1*, *osprr37*, *osprr59*, *osprr73*, and *osprr95* [10,12,13,15,17,39–41] mutants in the Nipponbare background used in the study were isolated in our laboratory using CRISPR/Cas9 technology.

2.2. Vectors' Construction and Transgenic Plants' Generation

To generate the overexpression construct of *DHD3*, the full-length coding sequence (CDS) of *DHD3* gene was amplified by specific primers, and the PCR (polymerase chain reaction) product was subcloned into binary vector *pCAMBIA1390* using an In-Fusion Advantage PCR Cloning Kit (Clontech, Beijing, China). *DHD3* was driven by the cauliflower mosaic virus 35S promoter. To knockout the *DHD3* gene using CRISPR/Cas9 technology, a 20 bp gene-specific sgRNA sequence of the target gene was cloned into the entry vector *pOs-sgRNA* and then subcloned into the destination vector containing the Cas9 expression cassette using the Gateway LR Clonase II Enzyme mix (Invitrogen, Shanghai, China) [42]. Primer sequences used for vectors' construction are listed in Supplementary Table S1.

The constructed vectors were introduced into the callus of rice variety Kitaake or Nipponbare by *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation [43]. To detect genotyping of transgenic plants, overexpressed plants are detected by amplifying the vector fragment inserted into the genome and agarose gel running. The mutant sites of genes from knockout plants were detected by amplifying flanking fragments containing the target site and then sending them to the company (Invitrogen, Shanghai, China) for Sanger sequencing. Primer pairs used for detection are listed in Supplementary Table S1.

2.3. RNA Extraction and RT-qPCR Assay

Total RNA was extracted from the leaves of plants using a ZR plant RNA MiniPrep Kit (ZYMO Research, Beijing, China) and reverse transcribed using a QuantiTect reverse transcription kit (Qiagen, Shanghai, China). RT-qPCR assay was performed using an SYBR premix Ex Taq Kit (Takara, Dalian, China) according to the user manual on ABI 7500 equipment. For normalization, the rice *Ubiquitin* (*UBQ*) gene was used as an internal control. All data were collected in biological triplicate and analyzed following the relative quantification method [44]. Primer sequences for RT-qPCR are listed in Supplementary Table S1.

2.4. Measurement of Agronomic Traits

The WT (wild-type) Kitaake and OE (overexpressed) lines were planted on 19 May 2016 and harvested on 3 May 2016. The WT Nipponbare and KO (knockout) lines were planted on 10 May 2017 and harvested on 9 May 2017. Multiple agronomic traits, including plant height, tiller number, panicle length, grains per panicle, primary branches number per panicle, secondary branches number per panicle, thousand-grain weight, and days to heading, were manually measured. The date of heading was recorded at the first panicle heading of about 1–2 cm of each plant and heading days were calculated by subtracting the seed sowing date [45]. The harvested seeds were air-dried in a glasshouse and oven-dried at 50 °C until ~14.0% moisture content remained when the weight was recorded as thousand-grain weight. Panicle length, number of primary branches, number of secondary branches, and number of grains per panicle were measured using the main panicles from WT and transgenic plants, respectively [46].

2.5. Subcellular Localization

The full-length CDS of *DHD3* was amplified and cloned onto *pAN580* vector while fusing the GFP (green fluorescent protein) tag at the C-terminal to generate DHD3–GFP. The DHD3–GFP fusion construct and marker vector (D53-mCherry) [47] were transiently

co-transformed into rice, as previously reported [48]. All images were generated by a ZESS LSM880 confocal microscope system.

2.6. Transactivation Activity Assay

Transactivation activity assay was performed by the Matchmaker GAL4 Two-Hybrid System (Clontech). The CDS of *DHD3* was cloned onto a vector (*pGBKT7*) while fusing the GAL4 DNA binding domain at the N-terminal. Then, the construction was transformed into the yeast strain AH109. The BD-Ehd4 was also transformed as a positive control [15], and the empty vector pGBKT7 (BD) was used as a negative control, respectively. The β-galactosidase activity was measured according to the Yeast Protocols Handbook (Clontech).

3. Results

3.1. DHD3 Is a Negative Regulator of Flowering Time and Has the Potential to Increase Yield in Rice

To explore new regulators of rice heading date, a transcriptional factor library was constructed using pUbi of maize as a promotor and VP64 (tetrameric repeats of VP16) fused by the rice transcription factors (TFs) as an activation domain. Hereafter, the library was introduced into the *japonica* rice cultivar Kitaake by an agrobacterium tumefaciens-mediated method and overexpressed transgenic plant lines were obtained [49]. So far, several genes regulating the rice heading date from the library have been identified and reported [20,50–55]. In this work, we identified six independent lines that contained the VP64-LOC_Os03g12370 sequence, and these transgenic lines delayed the heading date under both NLD and NSD conditions. Thus, we named *LOC_Os03g12370 Delayed Heading Date 3* (*DHD3*).

To verify the function of DHD3, we constructed a vector that overexpressed DHD3 under the control of pUbi promoter, and then transformed the vector into Kitaake. After two generations of plants, positive transgenic lines were obtained and confirmed by genomic PCR analysis. Three independent homozygous plant lines (DHD3–OE) showed high expression levels detected by RT-qPCR analysis (Figure 1A,B). Under the NLD condition, all three DHD3–OE lines delayed the heading date by about two weeks (Figure 1C). We selected one of the lines, DHD3–OE3, for further study. The plants of DHD3–OE3 grown in artificial light incubators showed a delayed flowering time under both LD and SD conditions (Figure 1D), whereas no significant difference in the leaf emergence rate of DHD3–OE3 and WT was observed before heading (Figure 1E,F). This suggested that the delayed flowering time of DHD3–OE3 plants was not due to retarded vegetative growth.

The overexpression of DHD3 has the potential to increase grain yield. The grains of the main panicle in DHD3–OE plants increased by about 77% (74.1%, 76.5%, and 81.7% for each line) compared with WT (Figure 2A,B), which suggests a corresponding increase in the potential yield. Besides, other agronomic traits such as plant height, panicle length, number of primary branches, and number of secondary branches were increased significantly (Figure 2C–F), though tiller number and thousand-grain weight did not change significantly (Figure 2G,H). Therefore, the increase in grain number per panicle was caused by the increase in the number of primary and secondary branches.

3.2. Gene Expression Analysis of DHD3

In order to investigate whether *DHD3* has a rhythmical expression pattern, the diurnal expression level of *DHD3* in rice leaves was examined by RT-qPCR every 4 h for 48 h under LD and SD conditions. The result showed that *DHD3* exhibited a similar rhythmical expression pattern of *DHD3* under both SD and LD conditions (Figure 3A,B). The transcription level of *DHD3* peaked at dawn and was relatively lowly expressed at dusk (Figure 3A,B). Besides, RT-qPCR analysis showed that *DHD3* was constitutively expressed in various rice tissues including the root, stem, sheath, leaf, and panicle, with the relative highest expression in sheath (Figure 3C).

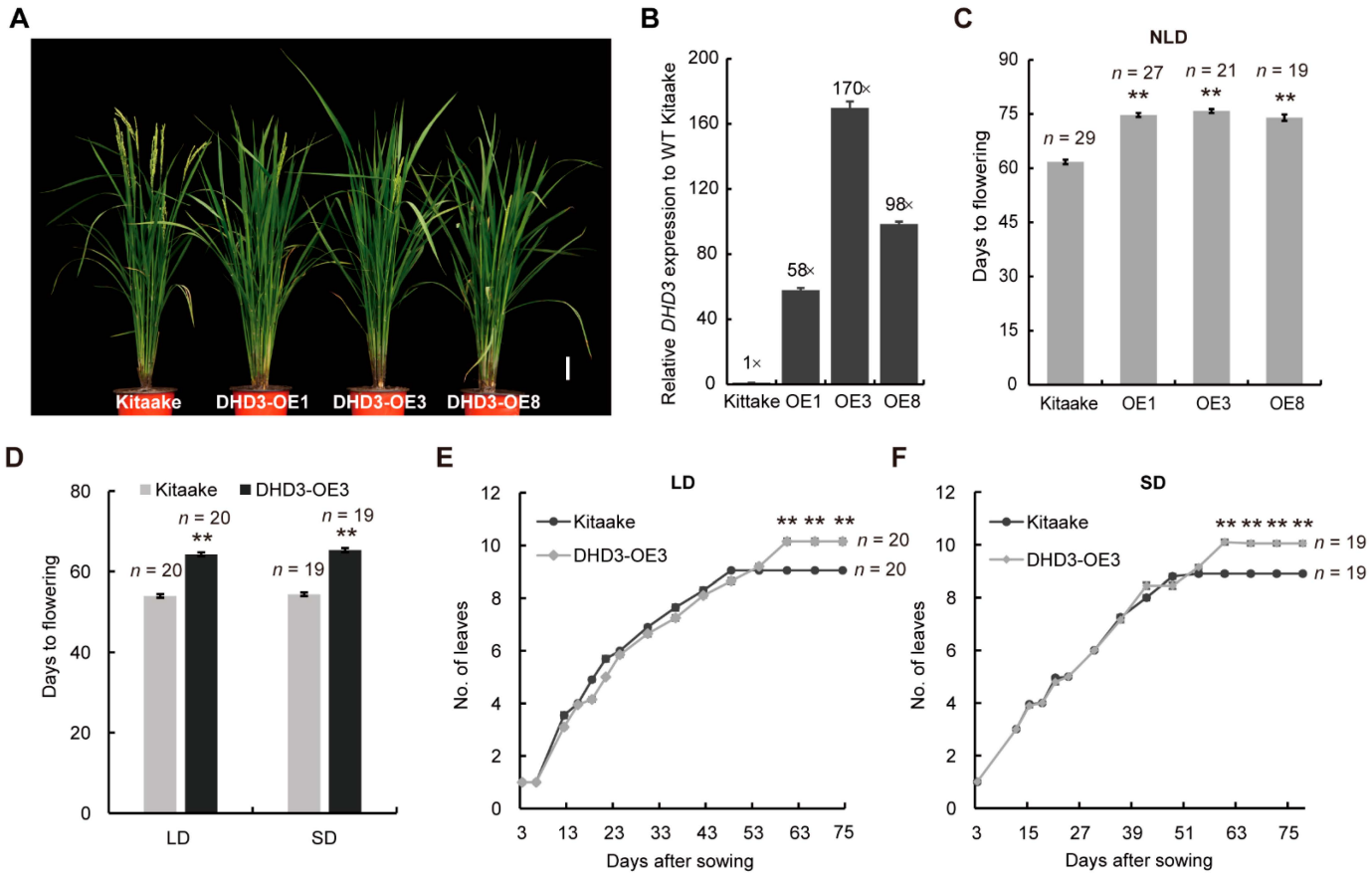

Figure 1. Phenotypic characterization of *DHD3* overexpressed (OE) plants. (**A**) WT and *DHD3* overexpressed plants that grew in a paddy field under the natural long-day (NLD) condition for 70 days. Scale bars, 5 cm. DHD3–OE1, DHD3–OE3, and DHD3–OE8, independent overexpressed lines of *DHD3*. (**B**) Expression levels in overexpressed plant lines. The labeled numbers are fold change of expression level relative to wild type (WT) Kitaake. (**C**) Heading dates of WT and *DHD3* overexpressed plants under the NLD condition. Means ± s.e. ($n > 15$). (**D**) Heading dates of WT and DHD3–OE3 overexpressed plants under control LD (long-day) and SD (short-day) conditions. Means ± s.e. ($n > 15$). (**E**,**F**) Leaf emergence rate of WT and DHD3–OE3 overexpressed plants under LD and SD conditions. Means ± s.e. ($n > 15$). *LSD*-test, ** $p \leq 0.01$.

3.3. DHD3 Protein Is Localized in the Nucleus and Has Weak Transactivation Activity

To investigate the subcellar localization of DHD3, the fusion protein DHD3–GFP and the nuclear marker (D53–mCherry) were transiently co-expressed in rice protoplast. The result showed that the green fluorescence signal exactly overlapped with the nuclear marker, indicating that DHD3 was localized in the nucleus (Figure 4A).

DHD3 belongs to the group of heat shock transcription factors (HSFs), which can regulate the expression of genes encoding HSPs by binding *HSEs* in their promoters. Thus, we performed the transcriptional activation assays of DHD3 in the yeast GAL4 system. The result showed that the yeast contains BD-DHD3 survive arduously on SD/-Trp-His-Ade medium (Figure 4B). Furthermore, β-galactosidase activity assay suggested a relatively weak transactivation activity of DHD3, as compared with transcriptional activator Ehd4 (Figure 4C) [15].

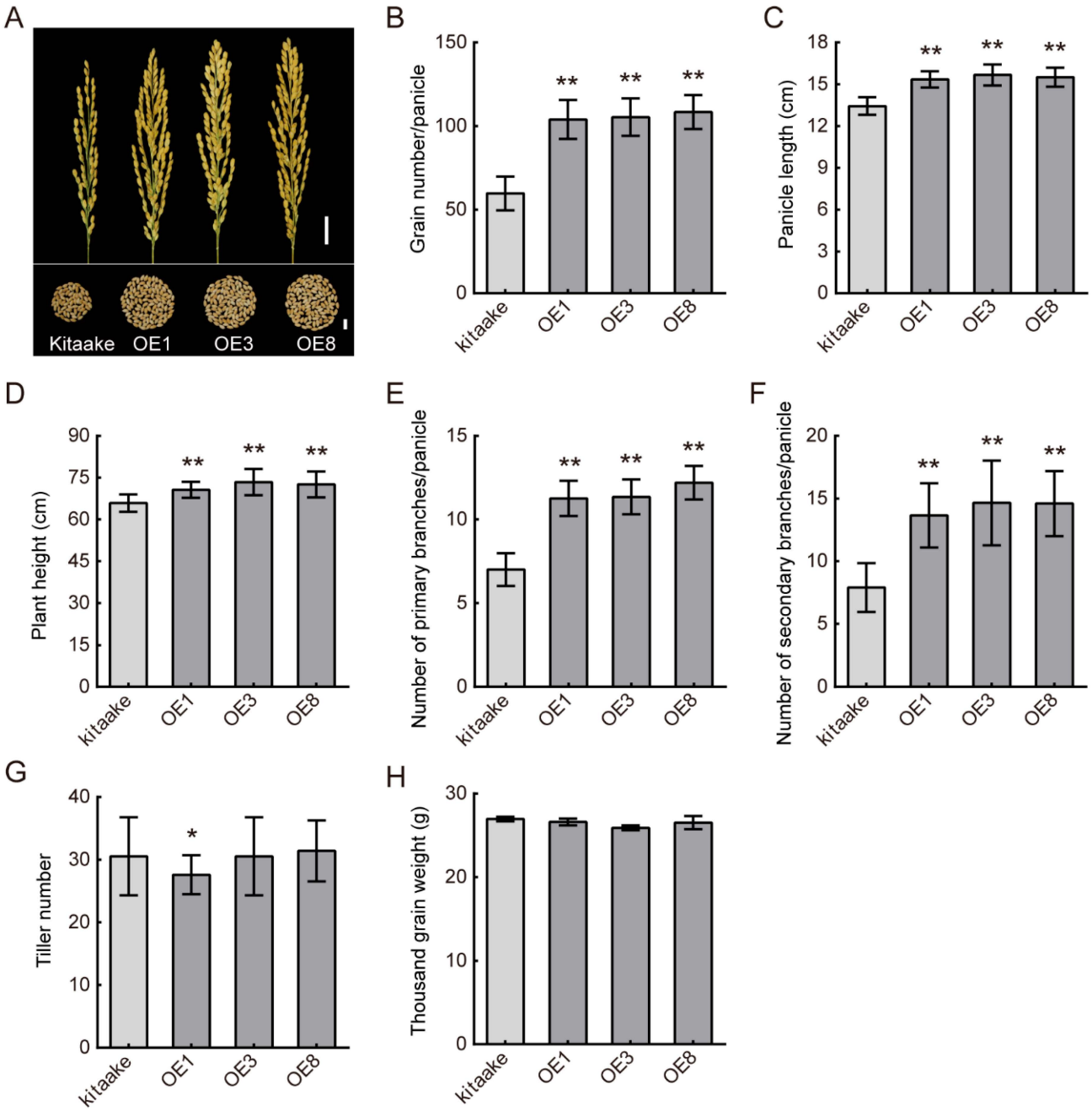

Figure 2. Agronomic traits of wild type Kitaake and overexpressed lines when planted in a paddy field under NLD conditions. (**A**) Main panicle size and grains on the main panicle of WT and *DHD3* overexpressed plants. Scale bars, 2 cm (above) and 10 mm (under). (**B**) Grain number of the main panicle. (**C**) The panicle length of the main panicle. (**D**) Plant height. (**E**) Number of primary branches of the main panicle. (**F**) Number of secondary branches of the main panicle. (**G**) Tiller number. (**H**) Thousand-grain weight. Data are means ± s.e. ($n > 15$). Statistical significance is indicated by * $p \leq 0.05$ and ** $p \leq 0.01$; one-way ANOVA test with Tukey correction.

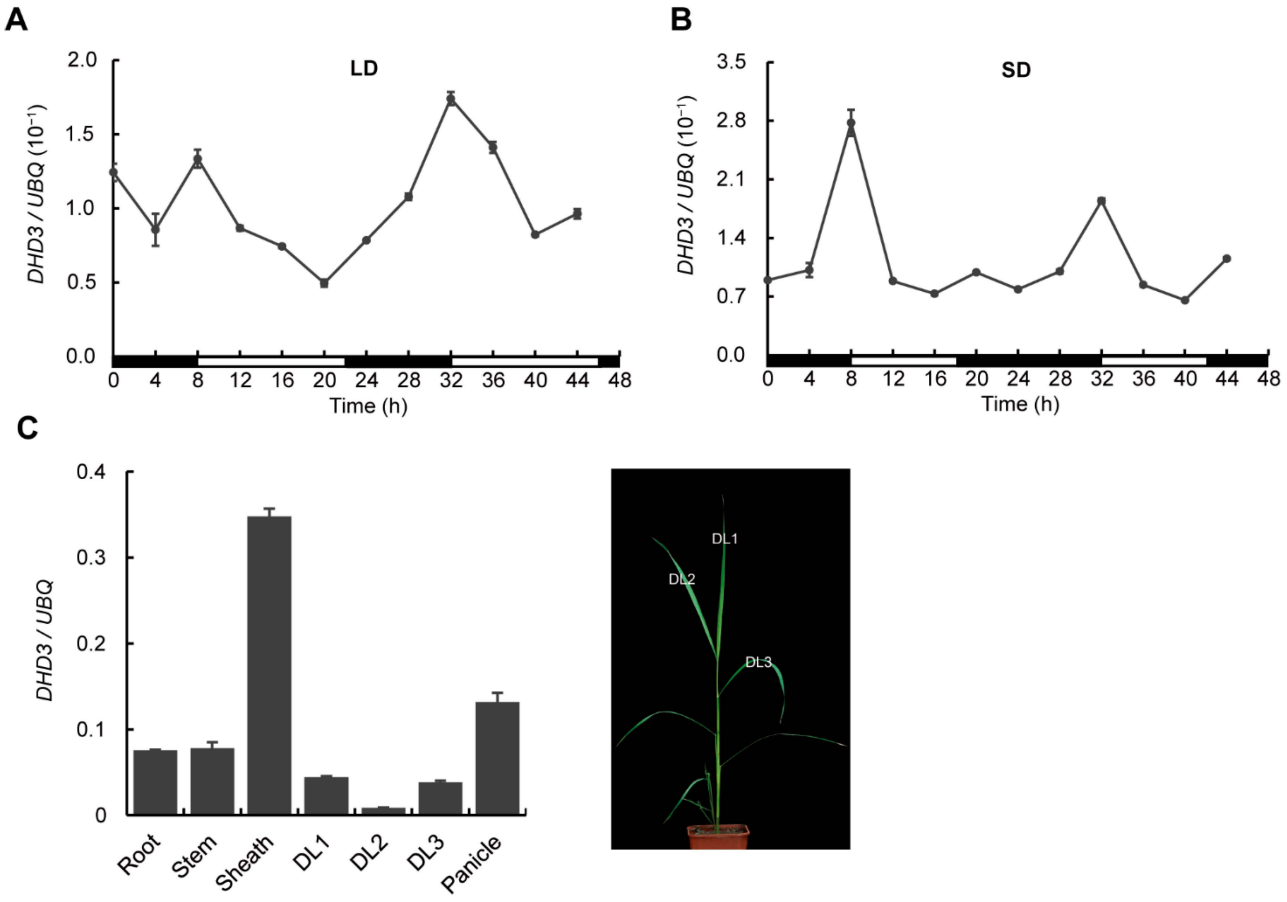

Figure 3. Expression patterns of *DHD3* by RT-qPCR analysis. (**A**,**B**) Diurnal expression pattern of *DHD3* under LD and SD conditions. Black and white boxes denote dark and light periods, respectively. (**C**) Relative expression of *DHD3* in different rice tissues, including root, stem, sheath, developed leaf (DL), and panicle. Data are means ± s.e. of three biological replications. Scale bars, 2 cm.

3.4. DHD3 Delays the Heading Date by Down-Regulating Hd3a and RFT1

To explore the downstream photoperiodic flowering genes regulated by DHD3, we examined the expression levels of rice flowering-related genes in DHD3–OE3 and WT plants by RT-qPCR. We found that the expression levels of *Hd3a*, *RFT1*, and *OsMADS14* are significant decreased in DHD3–OE3 plants under both LD and SD conditions (Figure 5A,B). Under the SD condition, *Ehd1* is expressed to a much lower extent in DHD3–OE3 plants than in WT plants at night, while *OsCOL4* increases compared with WT at dawn (Figure 5B). Under the LD condition, these two genes do not exhibit much change (Figure 5A). On the other side, there is not much change in the expression of *Hd1* under both LD and SD conditions (Figure 5A,B). This suggested that DHD3 suppresses the expression of *Hd3a* and *RFT1* to delay the heading date in rice, but may regulate flowering time by different pathways under LD and SD conditions.

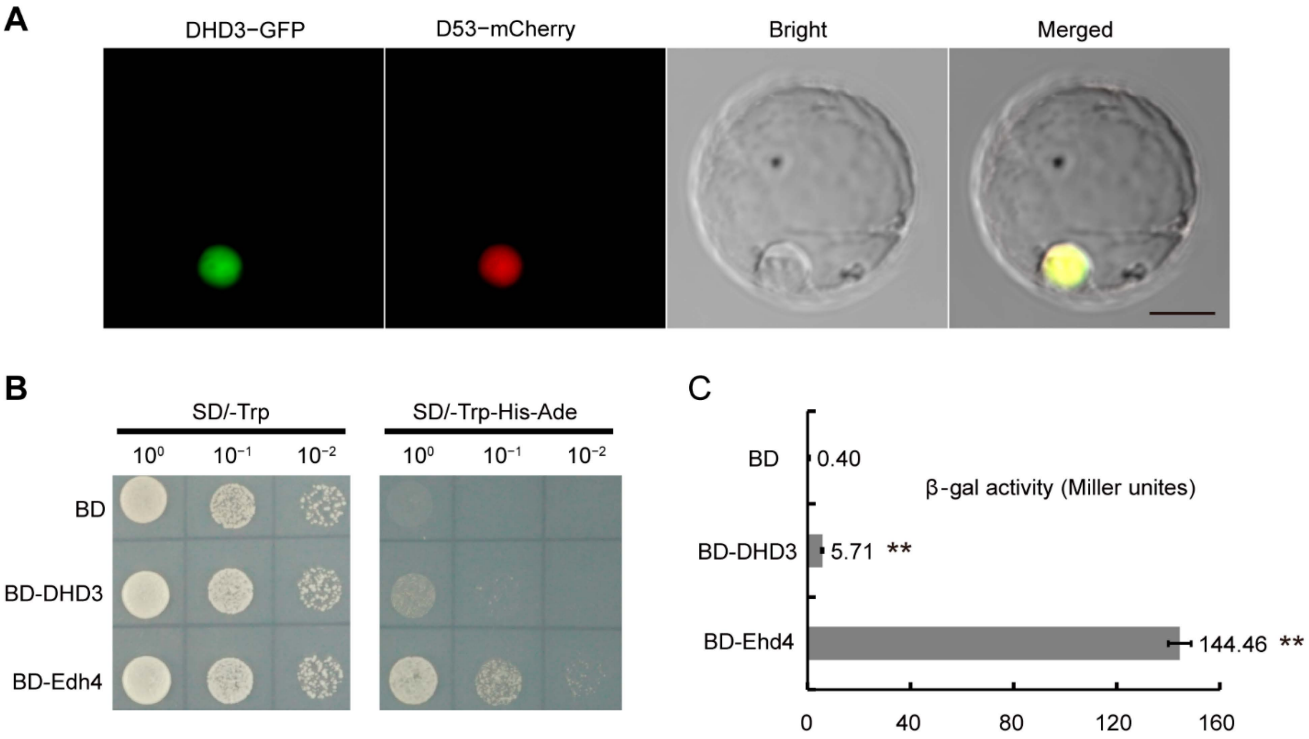

Figure 4. Subcellular localization and transcriptional activity of DHD3 protein. (**A**) Nuclear localization analysis of DHD3–GFP fusion protein in rice protoplasts by fluorescence microscopy. D53–mCherry was used as a nuclear marker. Bar, 10 μm. (**B**) Transcriptional activation assays of DHD3 in the yeast GAL4 system. The transformants were dropped onto SD/-Trp and SD/-Trp-His-Ade plates to grow for 48 h. 10^0, 10^{-1}, and 10^{-2} show the dilute fold of dripped yeast. (**C**) Values in β-galactosidase activity are means of three independent experiments. Data are means ± s.e. *LSD*-test, ** $p \leq 0.01$.

3.5. The Expression of DHD3 Is Independent of Some Other Heading Date-Related Genes, but Regulated by Circadian Rhythm-Related Genes

To study the role of *DHD3* in the regulatory network of rice heading date, we examined the expression level of *DHD3* in partial rice heading date mutants. The results show that the *DHD3* expression level does not much change in *ehd1*, *ehd2*, *ehd4*, *hd1*, *ghd7*, and *dth8* mutants (Figure 6A,B). On the other side, we found that *DHD3* expression was significantly impacted in some circadian rhythm-related gene mutants (Figure 6C,D). Under the LD condition, the expression of *DHD3* is more elevated in *osprr1*, *osprr37*, *osprr59*, *osprr73*, and *osprr95* (Figure 6C). In contrast, the *DHD3* expression is more decreased in these mutants under the SD condition (Figure 6D). These inconsistent results may be due to the different regulatory mechanisms under LD and SD conditions in rice. The exact role of *DHD3* in the rice heading regulatory network remains obscure and needs further in-depth exploration.

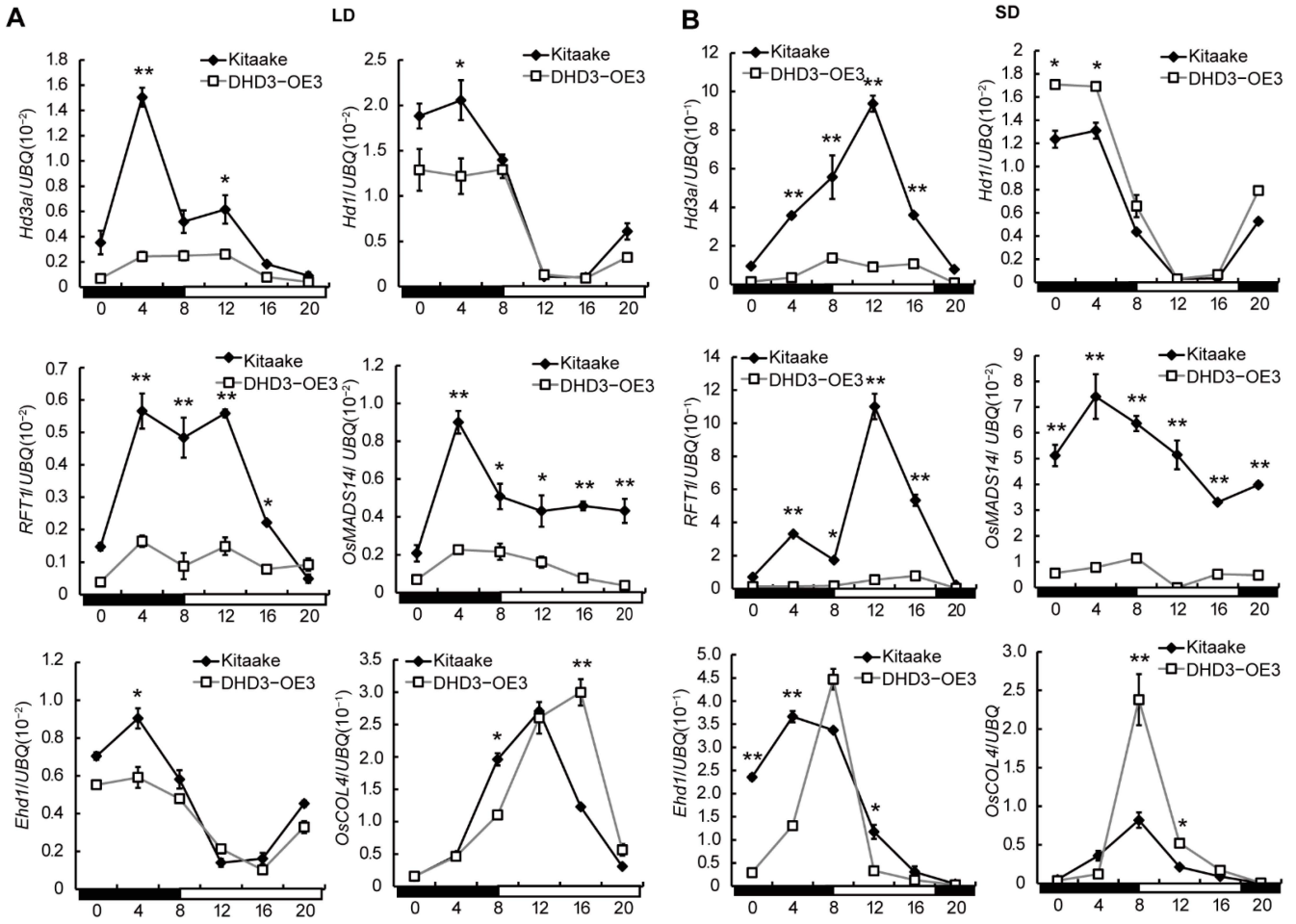

Figure 5. The expression levels of *Hd3a*, *RFT1*, *OsMADS14*, *Ehd1*, *OsCOL4*, and *Hd1* in WT (Kitaake) and DHD3–OE3 plants under LD (**A**) and SD (**B**) conditions. The black and white boxes denote dark and light periods, respectively. Data are means ± s.e. of three biological replications. Statistical significance is indicated by * $p \leq 0.05$ and ** $p \leq 0.01$; one-way ANOVA test with Tukey correction.

3.6. Suppression Effect of DHD3 on Heading Date Is Enhanced by Low Temperature

In order to validate whether DHD3 deficiency affects the heading date in rice, we generated *DHD3* knockout (KO) plant lines in the *japonica* rice Nipponbare using CRISPR/Cas9 technology. There is no difference in the heading date in two homozygous frameshift mutated *DHD3* KO (*dhd3-cr1*, *dhd3-cr4*) plant lines compared with WT (Figure 7A,B and Supplementary Figure S1). Considering that there are 26 HSF genes encoded by rice and 13 of them belong to Class A as DHD3 [56], it is likely that these genes share function redundancy and there are redundant genes that substitute the function of *DHD3*. It is no surprise that just knockout *DHD3* has no expected earlier heading date phenotype. Previous studies showed that *DHD3* expression is elevated in response to cold conditions [56]. To explore whether *DHD3* controls the heading date in rice under low temperature, we tested the cold response of the WT and DHD3–OE3 plants under cold conditions (constant 20 °C, SD). We found that, compared with normal temperature (SD, 30 °C, 10 h day/25 °C, 14 h night), WT Kitaake delayed flowering time for about 30 days, but DHD3–OE3 plants did not flower during the entire experimental duration (>163 days, the plants were grown in the incubator for too long to grow) (Figure 7C). This suggested that a low temperature can greatly enhance the flowering delay effect (delay 16.1% in 30 °C versus delay more than 89.3% in 20 °C) of DHD3 in rice. Meanwhile, both the WT Nipponbare and *dhd3-cr1* plants delayed flowering time by about 45 days under low temperature conditions, and there is no difference between these plants (Figure 7D).

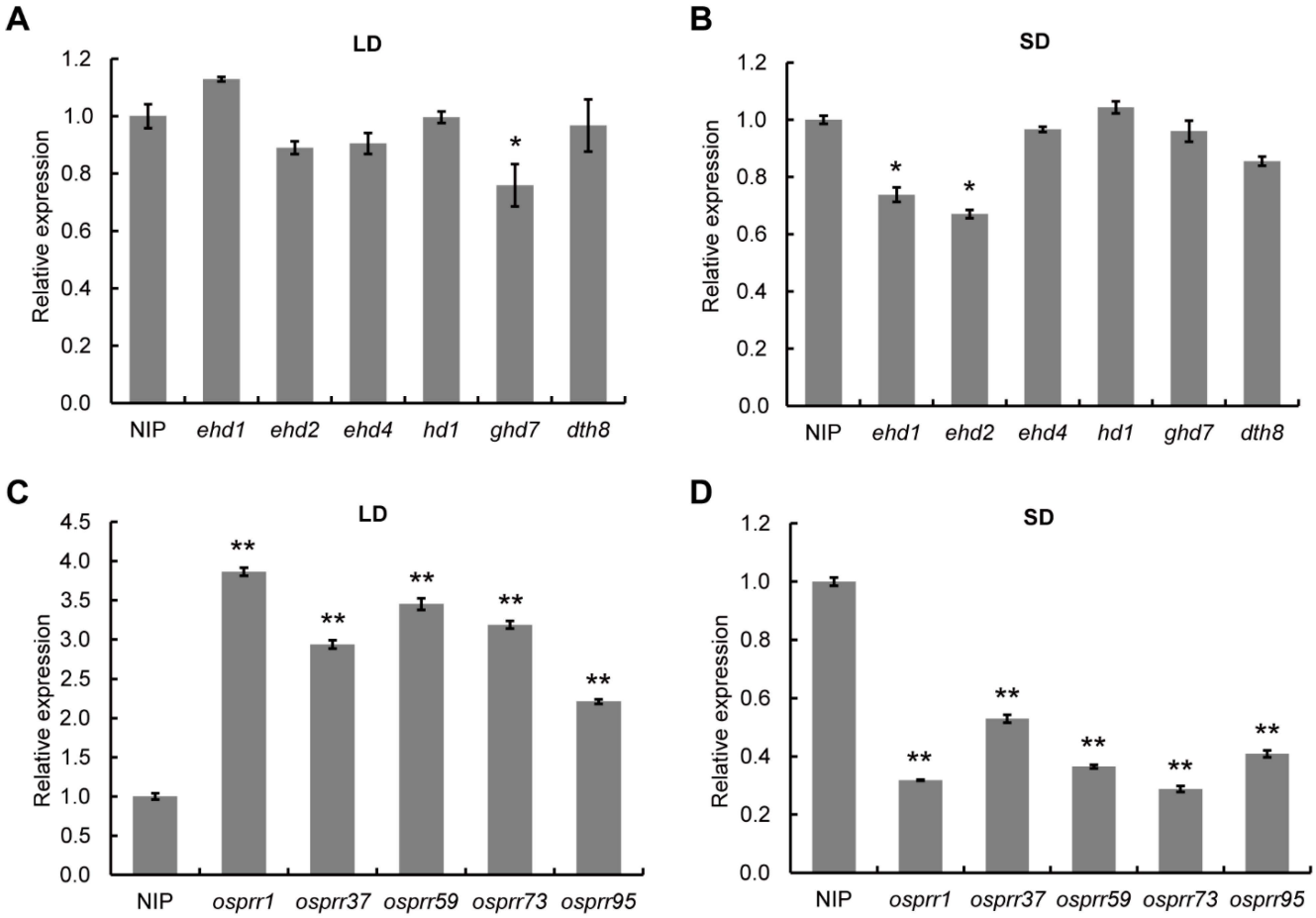

Figure 6. Expression analysis of *DHD3* in mutants related to heading stage. (**A**,**B**) *DHD3* expression levels in WT Nipponbare and *ehd1*, *ehd2*, *ehd4*, *hd1*, *ghd7*, and *dth8* mutants under LD and SD conditions. (**C**,**D**) *DHD3* expression levels in WT Nipponbare and *osprr1*, *osprr37*, *osprr59*, *osprr73*, and *osprr95* mutants under LD and SD conditions. Data are means ± s.e. of three biological replications. Statistical significance is indicated by * $p \leq 0.05$ and ** $p \leq 0.01$; one-way ANOVA test with Tukey correction.

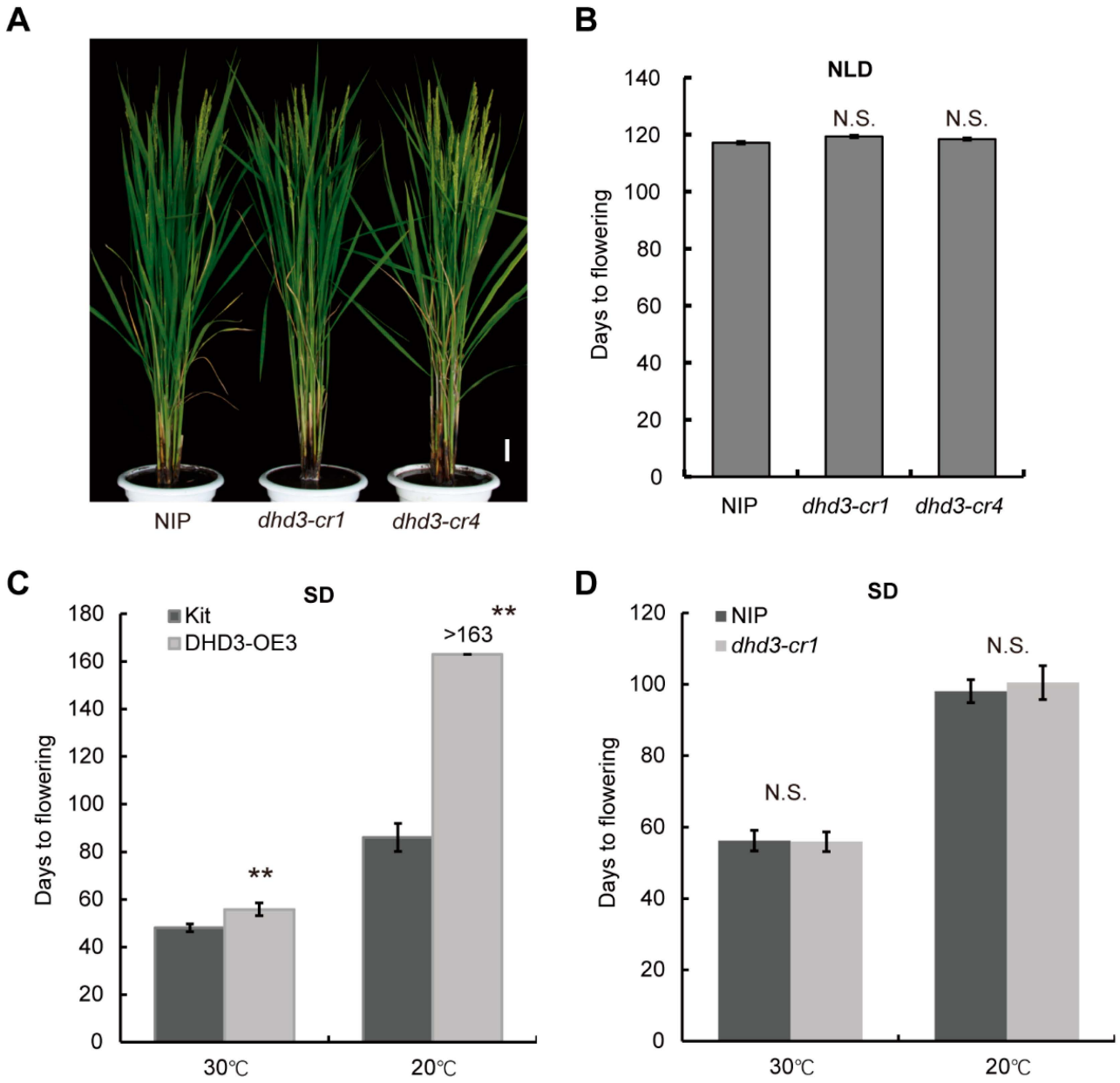

Figure 7. Effect of DHD3–OE plants and *dhd3-cr* mutant on heading date under low temperature conditions. (**A**) Phenotypes of WT Nipponbare (NIP) and DHD3 CRISPR-KO lines *dhd3-cr1* and *dhd3-cr4* grown in a paddy field under the NLD condition for 120 days. Scale bars, 5 cm. (**B**) Heading dates of WT NIP, *dhd3-cr1*, and *dhd3-cr4* grown in a paddy field under the NLD condition. Data are means ± s.e. ($n > 15$). N.S., not significant. (**C**) Heading dates of WT Kitaake (Kit) and DHD3–OE3 plants in 30 °C and 20 °C treatments under the SD condition. Data are means ± s.e. ($n > 15$). (**D**) Heading date of WT NIP and *dhd3-cr1* plants in 30 °C and 20 °C treatments under the SD condition. Data are means ± s.e. ($n > 15$). One-way ANOVA test with Tukey correction, ** $p \leq 0.01$. N.S., not significant.

4. Discussion

4.1. DHD3 Has the Potential to Increase Yield in Rice

HSFs are usually reported as stress-responding proteins. Here, we describe an HSF family gene *DHD3* that increases grain yield significantly by extending the vegetative period in rice for the first time. A delayed heading date in rice usually increases yield because it prolongs the vegetative stage of rice and allows the plants to accumulate higher

biomass before flowering [57,58]. However, too late of a heading date can cause the rice to experience low temperatures in reproductive growth, resulting in reduced yield. Therefore, it has been the goal of breeders to finely regulate the heading date of rice to adapt to different areas and cropping seasons and to achieve the maximum yield [59]. In our study, overexpression of *DHD3* in the *japonica* variety Kitaake delays the heading date and can increase the grains per panicle by 74%~81%, while the number of tillers and 1000-grains weight shows no significant changes (Figures 1 and 2). Hence, *DHD3* has the potential to increase yield in the rice breeding process.

4.2. DHD3 Is Involved in Temperature-Regulated Heading Date Pathway in Rice

HSFs have been widely studied in various species as a class of ubiquitous important environmental responsive transcription factors. HSFs have mainly been studied as a type of transcription factor that responds to various biotic and abiotic stresses in plant-related studies [29]. There are few reports that described the relationship between HSFs and plant development, such as flowering time. HSFB2B can bind to the promoter region and suppress the expression of *PRR7*. When *HSFB2B* is overexpressed, the circadian rhythm period of plants is changed under different temperatures and delays the flowering time in *Arabidopsis* [60]. The overexpression of *VsHSFA9* regulates *AtLED* expression, which is a GA signal pathway gene, and delays bloom in *Arabidopsis* [61]. Recently, HSFA2 was reported to regulate flowering time in *Arabidopsis* under heat stress. When plants are under heat stress, *HSFA2* is activated by H3K27me3 demethylation and upregulates the downstream gene *HTT5*, which induces earlier bloom in *Arabidopsis* [62]. So far, there is no HSF reported to be related to the heading date in rice. The heading date is delayed by low temperature in rice under both LD and SD conditions, and the expression levels of *Ehd1*, *Hd3a,* and *RFT1* are decreased under both day lengths [21,63]. However, *DHD3* (*LOC_Os03g12370*) expression is induced by cold treatment [56]. The promoter binding site assay shows that the binding site motifs of AP2s, DREBs transcriptional factors, which had been reported to be associated with cold response, were found on the promoter of *DHD3* [64]. Furthermore, the delayed heading date phenotype becomes much more dramatic in DHD3–OE3 plants under low temperatures (Figure 7C). These observations suggested that DHD3 may be a flowering time regulator that responds to a low-temperature environment in rice.

4.3. DHD3 May Be Involved in the Circadian Clock Signaling Pathway

The circadian clock is regulated by temperature rhythm through the HSF1 oscillation activity in mammals [65,66]. HSFB2B can bind to the promoter of circadian gene *PRR7* to regulate its expression [60]. Meanwhile, *DHD3* expression was significantly impacted in *osprr1*, *osprr37*, *osprr59*, *osprr73*, and *osprr95* mutants, which are circadian rhythm-related gene mutants. *OsPRR37*, *OsPRR59*, and *OsPRR73* have been reported to negatively regulate the heading date [67–69]. *OsPRR1* is a rice ortholog of the *Arabidopsis TOC1/PRR1* gene, which is a central element in one of the feedback loops of the circadian clock; it has been reported that it regulates panicle and grain size in rice [70]. In our work, the expression of *DHD3* is more elevated in these mutants under LD conditions (Figure 6C). In contrast, *DHD3* expression is more decreased in these mutants under SD conditions (Figure 6D), which implies that *DHD3* expression is regulated by these *OsPRR* genes under different light conditions. Hence, these results suggested that *DHD3* may also be involved in signaling pathways that cross-regulate circadian rhythms between photoperiod and temperature. The functions of *DHD3* in rice may be multiple and complex, and further research is needed.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture12071022/s1. Figure S1. Schematic diagram and Sanger sequencing of *DHD3*-knockout mutants. Table S1. Primers used in the study.

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Abbreviations

HSFs	heat shock transcription factors
DHD3	DELAYED HEADING DATE3
LD	long-day
SD	short-day
HSPs	heat shock proteins
HSEs	heat shock elements
DBD	DNA binding domain
OD	oligomerization domain
NLS	nuclear localization signal
NES	nuclear export signal
RD	repressor domain
ROS	reactive oxygen species
NLD	natural long day
UBQ	ubiquitin
NIP	Nipponbare
GI–Hd1–Hd3a	GIGANTEA–Heading date 1–Heading date 3a
PHYB	phytochrome B
Hd6	heading date-6
DTH8	days to heading on chromosome 8
Ghd7	grain number, plant height, and heading date 7
RFT1	RICE FLOWERING LOCUS T 1
Ehd2	early heading date 2
Ehd3	early heading date 3
Ehd4	early heading date 4
SDG724	SET domain group protein 724
OsCOL4	CONSTANS-Like 4
OsLFL1	O. sativa LEC2 and FUSCA3 Like 1
GA	gibberellic acid
DL	developed leaf
GFP	green fluorescent protein
PCR	polymerase chain reaction
KO	knockout
WT	wild-type
OE	overexpressed

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