Dlf1, a WRKY Transcription Factor, Is Involved in the Control of Flowering Time and Plant Height in Rice

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Abstract

Flowering time and plant height are important agronomic traits for crop production. In this study, we characterized a semi-dwarf and late flowering (dlf1) mutation of rice that has pleiotropic effects on these traits. The dlf1 mutation was caused by a T-DNA insertion and the cloned Dlf1 gene was found to encode a WRKY transcription factor (OsWRKY11). The dlf1 mutant contains a T-DNA insertion at the promoter region, leading to enhanced accumulation of Dlf1 transcripts, resulting in a semidominant mutation. The dlf1 mutation suppressed the transcription of Ehd2/RID1/OsId1 and its downstream flowering-time genes including Hd1, Ehd1 and Hd3a under both long-day (LD) and short-day (SD) conditions. Knock-down of Dlf1 expression exhibited early flowering at LD condition related to the wild-type plants. Accumulation of Dlf1 mRNA was observed in most tissues, and two splicing forms of Dlf1 cDNAs were obtained (OsWRKY11.1 and OsWRKY11.2). These two proteins showed transactivating activity in yeast cells. Dlf1 protein was found to be localized in the nucleus. Enhanced expression of OsWRKY11.2 or its 5’ truncated gene showed similar phenotypes to the dlf1 mutant, suggesting that it might function as a negative regulator. We conclude that Dlf1 acts as a transactivator to downregulate Ehd2/RID1/OsId1 in the signal transduction pathway of flowering and plays an important role in the regulation of plant height in rice.

Introduction

Increasing cereal output has been a fundamental goal to meet the soaring demand for food. Plant height, potential yield and flowering time are important traits for cereal production. Plant height, one of the main selection trait in rice (Oryza sativa) breeding, is controlled mostly by genes related to the synthesis and accumulation of phytohormones, such as gibberellin (GA) and brassinolide [1–3]. Potential yield per rice plant is determined by grain weight, and numbers of grains per panicle and tillers per plant [4–6]. Flowering time of plants is controlled by both environmental and developmental factors, with photoperiod as an important environmental signal. Molecular genetic analysis in Arabidopsis thaliana, a long-day (LD) plant, has identified a set of key regulators functionning in the photoperiod-mediated flowering pathway. For example, the nuclear protein CONSTANS (CO) positively regulates the flowering activator FLOWERING LOCUS T (FT), which further interacts with the bZIP transcription factor FLOWERING LOCUS D (FD) to control flowering time in Arabidopsis [7–9]. SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (SOC1), encoding a MADS box transcription factor, is activated by CO through FT and repressed by FLC (FLOWERING LOCUS C) via direct binding to the promoter [10].

Analysis of natural variants and mutants in rice, a short-day (SD) plant, has revealed the existence of a genetic pathway similar to that in Arabidopsis in photoperiodic flowering. Heading date 1 (Hd1), Heading date 3a (Hd3a), Heading date 6 (Hd6) and OsGI in rice are orthologs of Arabidopsis CO, FT, the α-subunit of kinase CK2, and GIGANTEA (GI), respectively. OsGI, a gene involved in the circadian clock control, regulates Hd1 and Hd3a in photoperiodic flowering, which promotes flowering under SD conditions and suppresses it under LD conditions [11–14]. In addition, early heading 1 (Ehd1), a B-type response regulator that is specific to floral induction in rice, regulates the expression of Hd1 and Hd3a in photoperiodic flowering [15]. Ehd1 functions upstream of Hd3a and RFT1 through the Hd1-independent pathway. Ehd2/RID1/OsId1 were isolated separately by three groups and the locus was found to encode a Cys2/His2-type zinc finger transcription factor orthologous to the INDETERMINATE 1 (ID1) gene in maize [16–18]. Loss-of-function ehd2/rid1/osid1 mutants were never- or extremely late-flowering in comparison with wild-type plants under both SD and LD conditions. Functional analysis revealed that Ehd2/RID1/OsId1 acts as a
master switch and promoter of phase transition mainly by regulating Ehd1 and the downstream genes. Further, a CCT (CO, CO-LIKE, and TIMING OF CAB1)-domain protein encoding gene Ghd7, which was uncovered from a natural variant rice, suppresses the expression of Ehd1 and Hd3a genes under LD conditions but does not affect Hd3a expression under SD conditions [19]. Recently, Hd17/Ef7, an ortholog of Arabidopsis EARLY FLOWERING 3 (ELF3), has been characterized to promote rice flowering by repressing Ghd7 expression under both LD and SD conditions [20,21]. In addition, Ehd3, which encodes a protein containing two plant homeodomain (PHD) finger motifs, also functions as a SD promoter functioning downstream of OsMADS51 and upstream of Ehd1 [9,26], whereas Ehd4, encoding a CCCH-zinc finger regulator, promotes rice flowering by stimulating the expression of Ehd1 and its downstream genes [27].

Much progress has been achieved in the genetic dissection of photoperiodic flowering of rice, but the molecular regulation is still largely unknown. In this study, we characterized a semi-dwarf and late flowering (dlf1) mutant and identified Dlf1 gene that encodes a WRKY transcription factor. Our results showed that overexpression of Dlf1 suppressed flowering by inhibiting the expression of Ehd2/RID1/OsId1 under both LD and SD conditions and influenced plant height in rice.

**Results**

**Isolation and morphological characterization of the dlf1 mutant**

A semi-dwarf and late flowering (dlf1) mutant was identified from a T1 population of T-DNA insertion lines of cultivar Oryza sativa L. japonica Zhonghua 11 (ZH11). The flowering time of dlf1 plants was delayed for about two weeks compared with wild-type plants in the experimental field in late May, 2003 in Hangzhou, China (Fig. 1A). DNA blot analysis showed that there was only one copy of T-DNA insertion in the mutant line (data not shown).

To determine whether the heading time of the dlf1 mutant differed under different photoperiod conditions, the mutant and wild-type plants were grown under SD conditions (10/14 h light/dark) and LD conditions (14/10 h light/dark). Heading time of the dlf1 mutant plants was 87.2±1.0 d, which was delayed ca. 16 d in comparison with wild-type (71.5±0.8 d) under SD conditions (Fig. 1E). Under LD conditions, heading time of the dlf1 mutant plants was 140.9±2.2 d and increased by ca. 23 d compared with ZH11 (117.8±2.2 d). Other phenotypes differed significantly under natural LD conditions, including homozygous dlf1 mutant lowered plant height (Figs. 1A and F) caused by reducing cell size (Fig. S1A), and decreased number of spikelets per panicle (Fig. S1B). The dlf1 mutant plants also had less 1000-grain weight (Fig. S1C) and showed leaf rolling phenotypes (Figs. S1D and E) under natural LD conditions.

**Dlf1 encodes a WRKY transcription factor**

Using the thermal asymmetric interlaced PCR (Tail-PCR) method, we isolated the genomic DNA flanking of the T-DNA insertion site from the dlf1 mutant. A BLAST search of the flanking sequence revealed that the T-DNA was inserted at 67 bp upstream of the initial ATG (the ‘A’ was defined as +1) of the predicted coding sequence of the gene OsWRKY11 (LO-
A

Insertion site: aaagaaga -42 bp deletion - cctctctca - 58 bp to the ATG

B

Primers
PddSalF and W6
TR1 and W6

C

ZH11  Xing-107

D

\[ Dlf1 \]

E

Plant height (cm)

\[ Xing107 \] \[ Xing137 \] \[ Xing137G \] \[ ZH11 \] \[ dlf1 \]

*
To ascertain whether the *dlf1* phenotypes are associated with the T-DNA insertion, genetic analysis was performed using two filial populations of reciprocal crosses between the *dlf1* mutant and wild-type plants. The flowering time and the plant heights co-segregated in a manner of fit the 1:2:1 population (data not shown). Correspondingly, the genotypes of the T-DNA insertion in the same populations were determined by PCR and showed a tight linkage with the phenotypes (Fig. 2B). These results indicated that the *dlf1* is semi-dominant and the T-DNA insertion event upstream of the *dlf1* gene is responsible for the mutant phenotype.

To examine the effect of the T-DNA insertion on the gene expression, the *Dlf1* total expression level was assayed by quantitative real-time PCR (qPCR). The transcript levels of *Dlf1* in rice leaves were enhanced significantly in the *dlf1* mutant compared with the wild-type ZH11 (Figs. 2D). To verify that the mutant phenotypes were caused by the high expression of *Dlf1* that was related to the T-DNA insertion, a fragment from the T-DNA insertion site to the end of the *Dlf1* coding region (*Cp-Ins-Dlf*) were transferred into the wild-type background. Among 14 plants regenerates named Xing-, 10 showed variation in dwarfism, and late flowering phenotypes in the T0 progeny (data not shown). Two lines were used for further analysis in the T1 generation. The levels of *Dlf1* expression were higher in dwarf plants compared with the wild-type and segregated non-transgenic plants (Figs. 2C, D and E). The dwarf plants also showed delayed flowering for about two weeks late under natural LD conditions, indicating that the increase of *Dlf1* expression caused the *dlf1* phenotypes.

**Dlf1 is ubiquitously expressed and alternatively spliced in rice.**

To examine the expression pattern of *Dlf1*, qPCR analysis was performed with total RNA prepared from leaves, shoots, young panicles and roots of ZH11 wild-type plants grown under natural LD conditions. *Dlf1* was expressed in all tissues examined (Fig. 3A). Further, we stained the transgenic plants harboring the *Dlf1* promoter- driven *Gus* fusion gene (−2010 to +134 bp; *Cp-WP:Gus*, Fig. 3B). Gus staining was observed in leaves, roots and panicles (Fig. 3C), which confirmed the qPCR results. Since the plants harboring *Cp-Ins-Dlf* construct showed the *dlf1* phenotypes, the fragment from the insertion site (−67 to +134 bp; *Cp-InsR3:Gus*) of *Dlf1* was also fused with *Gus* gene. Surprisingly, *Cp-InsR3:Gus* plants exhibited the similar level of expression as the *Cp-WP:Gus* plants in the young seedlings (Figs. 3D and E) though the *InR3* fragment was a region transcribed. However, the expression of *Gus* and *Gus* activity were low in the *Cp-InsR2R:Gus* (−67 to +5 bp) transgenic plants, which revealed that the deduced translation region in *InR3* was required for the promoter activity in comparison with the *In2R* fragment.

To obtain the *Dlf1* cDNA, we amplified the open reading frame region from total RNA isolated from ZH11 using RT-PCR with the primers W1 and W5. Two amplified products were obtained (Fig. 3F), the longer one (assigned as *OsWRKY11.1*) encoded the deduced 379-aa *Dlf1* protein and the shorter sequence (assigned as *OsWRKY11.2*) contained a 161 bp deletion in the third exon causing a premature stop of translation (Fig. S2). The deduced amino acid sequence of *OsWRKY11.2* encodes a protein of 270 residues, which still contains the WRKY domain (aa 205–262). Northern blot analysis revealed two hybridization bands using the *Dlf1* coding region, (Fig. 3G), confirming the existence of alternative splicing of the *Dlf1* mRNAs.

**Dlf1 has transcriptional activation activity in yeast.**

*Dlf1* contains an acidic N-terminus that may function as a transcriptional activation domain. To investigate this possibility, the coding region of the *Dlf1* full length cDNA and its truncated derivatives were fused in frame to the GAL4 DNA-binding domain in the pGBK7 vector. The transactivation activity assay in yeast showed that the region of 91–120 aa was required for its transactivation (Fig. 4). We also determined the activation activity of *OsWRKY11.2* (*pBD-WRKY11.2*), showing slight increase in activity compared with full length *Dlf1* transcript (*pBD-WRKY11.1*). This result was confirmed by deletion of the 3’ terminal of full length transcript (*pBD-dC2* construct).

Nuclear localization signal (NLS) of Dlf1 was predicted using nLS Mapper (http://nls-mapper.iab.keio.ac.jp/). A NLS (amino acid 151–181) was identified with a high score of 7.5. The *OsWRKY11.2* protein also contains the sequence of nuclear localization signal. To confirm the subcellular localization of Dlf1, we fused *OsWRKY11.1* with the enhanced green fluorescent protein (GFP) gene. The chimeric gene was put under the control of maize ubiquitin (*Ub*) promoter. The resulting plasmid (*Ub:Dlf1-GFP*) was then bombarded into onion epidermal cells. Localization of the WRKY11.1-GFP fusion protein was visualized exclusively in the nucleus (Fig. 5), whereas the control GFP (*Ub:GFP*) was distributed both in the cytoplasm and the nucleus, indicating Dlf1 is a nuclear protein.

**The expression of Ehd2, Ehd1, Hd1 and Hd3a was repressed in dlf1 mutant.**

To determine whether the late flowering phenotype of the *dlf1* mutant was due to the changes in flowering-related gene expression, qPCR analysis was performed in the wild-type and *dlf1* plants. Leaf samples were collected from 40 or 90-d-old plants grown under SD or LD conditions. The developmental stage of the plants was about one month before flowering in ZH11. The total expression levels of *Dlf1* in the mutant were higher than in the wild-type plants under both SD and LD conditions (Fig. 6). Moreover, *Dlf1* mRNA accumulation showed diurnal changes in the wild-type and *dlf1* mutant plants. *Ehd2/RID1/OsId1* is a key regulator in the genetic network that controls photoperiodic flowering in rice, promoting floral transition by upregulating Ehd1 and then the downstream *Hd3a* [15–17]. *Ehd2/RID1/OsId1* was...
mRNA accumulation was reduced in the \textit{dlf1} mutant compared with wild-type plants under SD and LD conditions (Fig. 6). Subsequently, the levels of \textit{Hd3a} and \textit{Ehd1} expression were decreased in the mutant plants. The expression of \textit{Hd1} was also suppressed in the \textit{dlf1} mutant under both SD and LD conditions (Fig. 6). However, the expression of photoperiod-related genes \textit{OsGI}, \textit{Se5} and \textit{Ghd7} were not significantly affected in the \textit{dlf1} mutant (Fig. S3), indicating that Dlf1 specifically suppressed the expression of \textit{Ehd2} and downstream genes.
Transcription levels of Dlf1 and the flowering-related genes were also examined at different developmental stages under LD conditions every 20 days. The accumulation of Dlf1 mRNA slightly increased and reached a peak at 70 d after germination. Subsequently, the transcript level gradually decreased and remained at low levels even after flowering in the wild-type plants (Fig. 7). In the dlf1 mutant, Dlf1 transcript accumulated in a pattern quite similar to that of the wild type ZH11 plants, but the expression levels of Dlf1 were at least 10-fold higher throughout the experiment periods.

Decrease of Dlf1 expression showed early flowering under LD conditions

To further dissect the function of Dlf1, several constructs were generated to examine possible roles of the different transcripts by means of overexpression and RNAi. The full-length OsWRKY11.1 and the alternatively spliced OsWRKY11.2 transcript were put under control of the Ubi promoter, respectively, generating the Ubi:W11.1 and Ubi:W11.2 constructs for rice transformation. To knockdown Dlf1, a 276-bp fragment was used for the RNAi construct under the control of the CaMV35S promoter. More than 15 independent overexpressing transgenic lines were obtained for Ubi:W11.1 in the genetic background of ZH11 and ZH17, respectively. However, only two Ubi:W11.1 lines of ZH17 genetic background (named as C-) were found to increase OsWRKY11 expression level and to delay flowering under LD condition comparing with ZH17 plants (Figs. 8A and B). Most of the transgenic lines in ZH11 background (named as OE-) showed slight variations in plant height (Fig. S4B) and insignificant changes in total transcripts of OsWRKY11 (data not shown). Further, RNAi lines of dch53 and dch57 displayed suppressed transcription of OsWRKY11 and flowered earlier than the ZH11 controls under LD condition (Figs. 8C and D). These results collectively suggested that Dlf1 negatively regulate flowering in rice.

The C-terminus of Dlf1 plays a role in the regulation of its expression level

In contrast to Ubi:W11.1 construct, most of the Ubi:W11.2 transgenic lines (named as Ka-) exhibited severe dwarfism (Fig. 9B).
and delayed flowering of about 2–3 weeks compared with ZH11 under natural LD conditions. The expression of total OsWRKY11 in transgenic plants was much higher than in ZH11 control and the segregated non-transgenic plants (Fig. 9C), suggesting that the C-terminus of Dlf1 plays a role in controlling its expression level.

Due to the promoter activity of InR3 fragment, transgenic plants were also obtained with the constructs excluding the N-terminal 37 aa of Dlf1 (assigned as Ubi:d4W11.1 and Ubi:d4W11.2). The Ubi:d4W11.2 transgenic lines (named as 4S-) presented dwarf and late flowering phenotypes under both LD and SD conditions (Figs. 9D and E), whereas the Ubi:d4W11.1 plants had no such phenotype (data not shown).

Analysis of the expression level of the transgenic plants revealed that the accumulation of OsWRKY11 total mRNA was increased over 400-fold in the Ubi:d4W11.2 progenies (Fig. 9F). These results suggest that the C-terminal region of Dlf1 influenced its expression and high level of expression of truncated OsWRKY11 might function in a similar manner as OsWRKY11.2.

To get a clue of the effect on Dlf1 expression, we examined the possible degraded mRNA of Dlf1 using the RNA ligase-mediated amplification of 5’ cDNA ends (RLM-RACE) [28]. A stronger PCR band was obtained in the dlf1 mutant than the ZH11 (Fig. 9G). Sequencing verified the degraded positions at 1093 and 1099 bp of Dlf1. These observations support the idea that RNA
as described in Fig. 1. Developing leaves were harvested 4 h after dawn. In ZH11 were scaled up 10 times. The plants were grown at conditions transcription levels in wild-type (filled squares) and are relative to the plants during development under LD conditions. The expression levels Changes of during development.

Discussion

In this study, we demonstrated that the Dlf1 gene had pleiotropic effects on a variety of traits, including flowering time, plant height, grain number and leaf rolling. Yield, plant height and heading date are the most important agronomic traits in rice, and a number of genes have been isolated that control each of these traits. For example, Gna1, a gene for cytokinin oxidase/dehydrogenase, regulates the number of grains per panicle [5], and a RING-type E3 ligase (GW2) controls grain width and weight [6]. The height of a rice plant is regulated by the gibberellin-insensitive gene Dwarf 1, encoding a CCT-domain protein, was shown to have multiple effects on grain number, heading date and plant height [19]. DTH8/Ghd8/Hd5, encoding a HAP3 subunit of the CCAAT-box binding protein, is also reported to suppress rice flowering under LD conditions and regulate plant height and yield potential [23,24]. Our data indicate that the rice WRKY transcription factor Dlf1 also widely affects rice development. Dlf1 regulates plant height by altering cell size in the internodes, similar to the effect of DTH8 but different from that of Ghd7 [Fig. S1] [19,23]. The phenotypes of short internode length and leaf rolling in the dlf1 mutant are supported by a recent report of OsWRKY11 transgenics, which is controlled by the promoter of heat shock-inducible HSP101 gene [29]. Among the four transgenic rice plants reported, three had bent leaves or dwarf phenotype, and two had significantly enhanced heat and drought tolerance under heat induction conditions.

The T-DNA insertion site in the dlf1 mutant was 67 bp upstream of the predicted translational starting site of OsWRKY11 (Fig. 2A). However, the Dlf1 expression was significantly increased in comparison with the wild-type plants. Transgenic plants harboring the genomic DNA of Dlf1, starting from the T-DNA insertion site to the end of Dlf1 coding region, recapitulated the dlf1 phenotypes (Fig. 2C), suggesting that the region has promoter function. This is confirmed by fusion with the Gus reporter gene (Fig. 3B, D and E). However, the Gus activities of Cp-WP:Gus and Cp-Inr3:Gus were at a similar level, inconsistent with a higher level of expression Dlf1 in the dlf1 mutant than that of the wild-type, implying a suppressor element existed outside of the WP fragment used in the experiment. We also generated Dlf1 overexpressing and RNAi transgenic lines. Unexpectedly, most of the OsWRKY11.1 transgenic plants did not show morphological differences to controls (Fig. S4). Nevertheless, two RNAi lines with decrease in OsWRKY11 expression showed early flowering under LD conditions (Fig. S8 and D). On the other hand, the accumulation of total OsWRKY11 mRNA (including the endogenous and transferred gene) was extremely high in the lines harboring the Ubi:W11.2 construct, which is 109 aa shorter than Ubi:W11.1 in the C-terminus (Fig. S2). These results suggested that the C-terminus of Dlf1 was involved in controlling accumulation level of its mRNA. This notion is further supported by comparison of the transgenic plants containing Ubi:d4W11.1 or Ubi:dhW11.2 constructs. Interestingly, most of the Ubi:W11.2 and Ubi:dhW11.2 transgenic plants exhibited dwarf and late flowering phenotypes, similar to the dlf1 mutant (Fig. 9). Likely, OsWRKY11.2 retained the transactivating activity (Fig. 4) and the sequence of nuclear localization signal (position 179–187 aa). The results suggested that the high level of OsWRKY11.2, or its N-terminus-truncated protein might function as a negative regulator. This information also implies that different spliceosomes of Dlf1 might work together to regulate downstream gene expression, although further study is required to test the existence of alternative splicing in planta.

RNA processing plays an important role in control of plant flowering time. FLOWERING LOCUS C (FLC), a repressor of the transition to flowering in Arabidopsis, functions to delay flowering by down-regulating expression of genes promoting the floral state. Processing of FLC mRNA is regulated by autonomous pathway components of FCA and FY, which encode a pre-mRNA processing protein and a homolog of the yeast RNA 3’-processing factor Psp2p, respectively [30,31]. FCA expression is also regulated through alternative processing of its pre-mRNA into four different transcripts, in which only the fully spliced FCA transcripts can promote flowering [30]. Furthermore, FCA negatively regulates its own expression by increasing cleavage and polyadenylation within

processing proteins or microRNAs may regulate the expression of Dlf1, if the level of Dlf1 mRNA containing the 3’ end reached a limit.

Discussion

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intron 3, thus limiting the production of active FCA protein to keep the balance of pathways controlling flowering time. They also found that active FCA can be overexpressed only when the cis-element within intron 3 required for the negative feedback is removed [32,33]. As mentioned above, the accumulation of OsWRKY11.1 mRNA was not much increased in the overexpressing plants. An explanation is that the C-terminal part of OsWRKY11.1 interacts with a protein involved in regulation of the OsWRKY11.1 mRNA level. When the OsWRKY11.1 protein reaches a threshold level it will activate the protein–protein interaction and decrease accumulation of the OsWRKY11.1 transcript. This or another interaction might also possibly involve in the alternative splicing of Dlf1.

Rice is a facultative SD plant which flowers under LD conditions. As a counterpart of the G1-CO–FT signaling pathway in Arabidopsis, the rice orthologous proteins consist of OsGI–Hd1–Hd3a. The clock-associated protein OsGI upregulates Hd1 expression and in turn Hd1 induces the expression of Hd3a during SD conditions in rice [9,12,13]. The expression of Hd3a is also induced by the Ehd1 activator, which is an evolutionarily unique gene in rice with no counterpart in Arabidopsis [15]. Ehd2/RID1/OsId1 was found to promote flowering under both SD and LD conditions by upregulating Ehd1 expression [16–18]. Since Ehd2/RID1/OsId1 expression was suppressed under both SD and LD conditions in the dlf1 mutant (Fig. 6), the late flowering phenotype of the mutant is easily explained by loss of the promoting action of Ehd2/RID1/OsId1 (Fig. 9).

Dlf1 was expressed in leaves, roots and panicles (Fig. 3A). Expression of Dlf1 in leaves is consistent with the role of genes in flowering-time regulation, such as Ehd2 and Ghdf7 [16,19]. Ehd2/RID1/OsId1 is considered the master switch for the transition from vegetative to reproductive phase, a crucial process in higher plants. We found that increased Dlf1 expression delayed the phase transition and initiation of floral induction, leading to late flowering in the dlf1 mutant. This was further supported by studies of gene expression in the whole developmental process, which showed that Ehd2/RID1/OsId1 is suppressed in the dlf1 mutant with a higher level of Dlf1 mRNA accumulation under LD conditions (Fig. 7). Diurnal expression of Dlf1 was observed under both LD and SD conditions; however, the expression of photoperiod-related genes OsGI and Se5 was not significantly changed between the mutant and wild-type plants (Fig. 6; Fig. S5), suggesting that Dlf1 is unlikely to be upstream of these genes in the pathways of photoperiodic flowering in rice. WRKY proteins are a super family of plant transcription factors, which are characterized by binding specifically with W-box (a core sequence of TGAC).

Our data combined with others indicate that Dlf1 play important roles on plant height, heading date, yield potential and responses to abiotic stress [29]. The CCT-domain protein Ghdf7 and DTH8 protein have been demonstrated to have pleiotropic effects on heading time, height and yield potential [19,23]. Further investigation of their relationship should help illuminate the complexities of these important agronomic traits, as well as aid in manipulation of the traits for yield improvement.
Figure 9. High level expression of the OsWRKY11.2 leads to dwarfism and late flowering. (A) Schematic diagram of Ubi:W11.2 (Ka-) and Ubi:d4W11.2 (4S-, with 37-aa deletion at the N-terminus of W11.2) constructs. (B) and (D) Plant heights of those transformed with Ubi:W11.2 or Ubi:d4W11.2 in ZH11 or ZH17 genetic background, respectively. (C) Expression of total OsWRKY11 (including the transferred and endogenous genes) in ZH11 and the Ubi:W11.2 transgenic lines of T1 progenies under natural LD conditions. The first and second youngest leaves were sampled from 90-d-old plants for RNA isolation. Transcription levels were quantified by qPCR and the gene expression was normalized to rice ubiquitin gene (Ubq) for each sample. Transcription levels are expressed as ratio to the level of transcript in ZH11. The suffix A for dwarf and G for segregated non-transgenic plants. (E) Days to heading of the Ubi:d4W11.2 plants of T2 progenies under SD and LD conditions (the same treatments as in Fig. 6). (F) Expression of total OsWRKY11 in ZH17 and the Ubi:d4W11.2 transgenic plants of T2 progenies under both LD and SD conditions (the same treatments as in Fig. 6). Transcription levels are expressed as ratio to the level of transcript in ZH17. (G) Analysis of the possible degraded mRNA of Dlf1 using the RNA ligase-mediated amplification of 5’ cDNA ends (RLM-RACE). Total RNAs were isolated from the dlf1 mutant (dlf1) and the wild-type (ZH11) of three-week-old plants. Two rounds of PCR were performed: 1) by using an outer primer from the adaptor and a Dlf1-specific reverse primer W5; and 2) by using the inner primer from the adaptor and a Dlf1 gene primer W4 for 30 cycles. Actin gene was used as an internal standard. Asterisks indicate significant difference between ZH11 and the overexpression lines (P<0.05, Duncan test). a, b, c, d, and e indicate ranking by Duncan test at P<0.05, starting from a. Different letters indicate significantly difference from each other.

doi:10.1371/journal.pone.0102529.g009
Materials and Methods

Plant materials and growth conditions

Rice seeds of the wild-type (Oryza sativa L. japonica, Zhonghua 11 or Zhonghua 17, ZH11 or ZH17), mutant and transgenic progenies were sterilized and germinated on half-strength Murashige and Skoog medium for 7 d and then transferred to soil in a greenhouse. For flowering time measurements, plants were grown either in 10/14 h light/dark for SD or 14/10 h light/dark for LD. Rice flowering time was measured in days from germination until emergence of the first panicle. For diurnal expression analyses, young leaves were harvested from wild-type ZH11 and the dlf1 mutant of 40-d-old (SD) or 90-d-old (LD) plants at 4-h intervals for a total of 24 h. To analyze gene expression during development, the first and second youngest leaves from three plants were harvested from 30, 50, 70, 90, 110 and 130-d-old plants at 4 h after dawn under LD condition. The rice plants examined under natural field conditions were sown at late April and transplanted at early June in the experimental field of China Agricultural University, Beijing (39°54′N, 116°23′E), China.

Genotyping of mutant plants and Tail-PCR

For genotyping analysis, the dlf1 segregating population was assayed by PCR using the primers of PddSalF, TR1 and W6 (Table S1). The primer pair of TR1 and W6 was used for amplification of the T-DNA insertion. Thermal asymmetric interlaced PCR (TAIL-PCR) method was used to isolate genomic fragment flanking the T-DNA insertion site from the dlf1 mutant plant. The primers TR1, TR2, TR3, AD1, AD2 and AD3 are shown in Table S1.

Vector construction and transformation

The full-length coding region of Dlf1 was obtained by PCR amplification using the primers W1 and W5, along with a shorter product (assigned as OsWRKY11.2). The OsWRKY11.1 and OsWRKY11.2 cDNAs were put under the control of a maize ubiquitin promoter in a modified pambia 1301 vector to generate Ubi:W11.1 and Ubi:W11.2 for overexpression [34]. Similarly, the PCR products, amplified with the primer pairs of d10BIF/W10H3r and d10BIF/W10H3r were used to construct Ubi:d4W11.1 and Ubi:d4W11.2 vectors, with deletions of the 5′-ends in comparison with OsWRKY11.1 and OsWRKY11.2, respectively. Each overexpressing construct contained a Flag tag in the 5′-end of the gene. The Dlf1 fragment of 276 bp (from −39 to +237 bp) was used to generate the Dlf1:RNAi plasmid using procedures similar to the previous description [34]. The hairpin structure was put under the control of the CaMV35S promoter (CaMV35S:dsW11). For promoter constructs, the PCR products were fused to β-glucuronidase (Gus) reporter gene as following: the Dlf1 promoter from −2010 to +134 bp as Cp-WP:Gus, −67 + 134 bp as Cp-InR3:Gus, and −67 + 5 bp as Cp-In2R:Gus. For complementation, the genomic DNA fragment from −67 to the end of Dlf1 was put into a modified pambia 1301 vector, designed as Cp-Ins-Dlf. All vectors were verified by sequencing and transformed into rice through Agrobacterium-mediated transformation [35]. The transgenic lines obtained were determined by PCR amplifications.

Transactivation activity assay

The coding region of Dlf1 was amplified with the primers W10E1 and W10BgSal (Table S3). The PCR product was fused to the GAL4 DNA binding-domain vector pGBK77 (pBD, Clotech) to generate the plasmid pBD-Dlf1. Similarly, a fragment of Dlf1 encoding amino acids 91-379 (pBD-dN3), 121-379 (pBD-dN2), 204-379 (pBD-dN3), 91-313 (pBD-dN1), the first 313 amino acids (pBD-dC1), the first 266 amino acids (pBD-dC2), or OsWRKY11.2 (pBD-WRKY11.2) were fused to the GAL4 DNA binding-domain. These constructs or empty vector pBD were individually transformed into yeast cells of AH109 strain and grown on SD–Trp selective medium at 30°C for 3 d. An assay of β-galactosidase activity was performed with transformed cell lines grown in liquid SD–Trp medium using o-nitrophenyl β-D-galactopyranoside as a substrate, according to the manufacturer’s protocol.

Subcellular localization of Dlf1

The coding sequence of Dlf1 was amplified and fused in frame to the upstream of green fluorescent protein (GFP) gene to generate the CamUbi-Dlf1-GFP construct. The resultant and the control CamUbi:GFP vectors were transformed into onion (Allium cepa) inner epidermal cells by bombardment using the PDS-1000/He system (Bio-Rad) with DNA-coated gold particles. The transformed cells were cultured on 1/2 MS medium at 26°C for 2 d and observed under a confocal microscope (Bio-Rad MRC 1024).

Gus assay and histochemical staining

Gus activity assay and histochemical staining were performed as described [45] and photographed using a Nikon SMZ 1000 stereo microscope with a Nikon digital sight DS-SM camera or Nikon camera.

RNA gel-blot

Trizol reagent was used to extract the RNA from rice tissues. Total RNA was fractionated in 1.5% agarose containing formaldehyde, blotted onto Hybond-N nylon membrane and probed with the coding sequence of Dlf1. Hybridization was performed as previously described [34] and the membrane was autoradiographed by using a phosphoimaging system (Amersham Pharmacia Biotech, UK).

Real-time quantitative RT-PCR

Total RNA was isolated from different rice tissues using Trizol reagent following the manufacturer’s procedures. DNaseI-treated RNAs were reverse transcribed with oligo (dT) and random primers. Real-time quantitative RT-PCR (qPCR) was performed in a final volume of 20 μL, including 10 μL SYBR Premix EX Taq (Takara), 2 μL of the diluted first-strand cDNA as templates and 0.2 μM of each primer. The reactions were carried out with an ABI PRISM 7000 or ABI StepOne in the following program: 95°C for 2 min, 40 cycles of 95°C for 5 s, 60°C for 20 s, and 72°C for 31 s. Every experiment was repeated more than twice. The primers of Ehd2, Hld1, Ehd1, Hld3a, Ubq, Ghd7, OsG1, Se5, FTL6 and Dlf1 are listed in Table S2. The level of ubiquitin (Ubq) expression was used to normalize the expression ratio of each gene.

RLM-RACE PCR

The RNA ligase-mediated amplification of 5’ cDNA ends (RLM-RACE) was performed according to the manufacturer’s manual with modifications. Two μg total RNA was directly ligated to a RNA adaptor using T4 RNA ligase and transcribed by random primers. PCR was performed by using an outer primer from the adaptor and a Dlf1-specific reverse primer W5 at conditions of 95°C for 3 min (1 cycle), 95°C for 30 s, 55°C for 30 s, 72°C for 1 min (20 cycles), then 72°C for 10 min (1 cycle). A second round of nested PCR was amplified by using the inner
primer and a Dlf1 gene primer W4 for 30 cycles. Actin gene was used as an internal standard. The RACE PCR products were sequenced to identify the mRNA degraded ends.

Supporting Information

Figure S1 Phenotypes of Zhonghua 11 (ZH11) and dlf1 mutant. (A) Longitudinal section of the stems approximately 2 cm above the upper-most nodes from the tiller culms of plants. (B) Number of spikelets per panicle. Values are means ± SD, n = 20. (C) 1000-grain weight. Values are means ± SD, n = 10. (D) Leaf rolling. (E) Transverse sections of the middle part of the first leaf from tillering plants. The plants were grown in the experimental field under natural LD conditions. (PDF)

Figure S2 Multiple sequence alignment of OsWRKY11 cDNAs and proteins. (A) The full-length cDNA of Dlf1 (OsWRKY11.1) and the alternatively spliced transcript OsWRKY11.2 were aligned using the CLUSTAL W program. (B) The alignment of Dlf1 and OsWRKY11.2 proteins using CLUSTAL W program. (PDF)

Figure S3 Expression of OsGI, Ghd7, Se5, and FTL6. Diurnal expression patterns of OsGI, Ghd7, Se5, and FTL6 in the ZH11 control (filled circle) and the mutant dlf1 (open circle) plants under SD (10 h light/14 h dark) and LD (14 h light/10 h dark) conditions by qPCR analysis. The expression levels are relative to the ubiquitin (Ubg) mRNA. Values are shown as means ± SD of two independent experiments. The open and filled bars at the bottom represent the light and dark periods, respectively. (PDF)

Figure S4 Heights of Ubi:W11.1 transgenic plants. (A) Schematic diagram of Ubi:W11.1 construct (OE-). (B) Plant heights of some Ubi:W11.1 lines in T2 progenies and the ZH11 control. Values are means ± SD. (PDF)

Table S1 Primers of Dlf1 for genotype, expression, and vector construction. (DOCX)

Table S2 Primers of photoperiod- and flowering-time-related genes for real-time RT-PCR. (DOCX)

Table S3 Primers for transactivation activity. (DOCX)

Acknowledgments

The authors thank Professors Yiji Xia (Hong Kong Baptist University) and Jun Fan (China Agricultural University) for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: YC XC ZG. Performed the experiments: YC XC KX QX YW JL CD. Analyzed the data: YC XC ZG. Contributed reagents/materials/analysis tools: ZS. Contributed to the writing of the manuscript: YC XC ZG.

References


