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JcDof1, a Dof transcription factor gene, is associated with the light-mediated circadian clock in *Jatropha curcas*

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Jatropha curcas is an economically important plant in terms of its seed oil. However, the molecular mechanisms underlying this plant response to light signals are unknown. One group of DNA-binding with one finger (Dof) transcription factor genes exhibits circadian rhythms and plays a crucial role in the control of flowering time by photoperiod perception in plants. In the present study, a full-length cDNA designated *JcDof1,* containing a conserved Dof-DNA-binding domain, was isolated from *J. curcas* seedlings by yeast one hybrid library. Subcellular localization assays and yeast one hybrid systems confirmed that JcDof1 was localized to the onion epidermal cell nucleus, and exhibited DNA-binding and transcriptional activation activities in yeast. The *JcDof1* expression was characterized by a circadian-clock oscillation under long day, short day and continuous light conditions, whereas in the etiolated cotyledons under continuous dark conditions, *JcDof1* expression remained at relatively basal levels. Red and blue light downregulated the *JcDof1* expression, but this effect was not observed under far-red light. Taken together, these results suggested that *JcDof1* was a circadian clock-Dof transcription factor gene responding to light signals.

Introduction

DNA-binding with one finger (Dof) domain proteins are a group of plant-specific transcription factors with a single Cys2/Cys2-type zinc-finger-like motif (Lijavetzky et al. 2003, Yanagisawa 1995, 2002). The highly conserved Dof domain which presumably includes 52 amino acids, specifically recognizes an AAAG DNA-binding core sequence (or its reversibly orientated sequence, CTTT) and mediates other protein–protein interactions (Imaizumi et al. 2005, Yanagisawa 2002, Yanagisawa and Sheen 1998). Since the first *Dof* gene (*ZmDof1*) was isolated from maize (Yanagisawa and Izui 1993), the discovery of many putative *Dof* genes was reported in both monocots and dicots, including 37 genes in *Arabidopsis thaliana* (Riechmann et al. 2000, Sawa et al. 2007, Yanagisawa 2002), 30 genes in *Oryza sativa* (Lijavetzky et al. 2003), 26 genes in *Hordeum vulgare* (Moreno-Risueno et al. 2007) and 28 genes in soybean (Wang et al. 2007). The presence of many *Dof* genes indicates that multiplication of *Dof* genes might be linked with the evolution of sophisticated transcriptional control in plants. Several Dof proteins have been found to play diverse roles in the gene regulation of many processes. For instance, maize ZmDof1 is involved in light-regulated gene expression and distinct activities in the greening and the etiolated protoplast. Both ZmDof1 and ZmDof2 specifically interact with the promoter of

Abbreviations – B, blue; *CO*, *CONSTANS*; CT, comparative threshold; DD, continuous dark; Dof, DNA-binding with one finger; FR, far-red; LD, long day; LL, continuous light; Q-PCR, quantitative real-time polymerase chain reaction; R, red; SD, short-day or synthetic dropout; ZT, *zeitgeber* time.

phosphoenolpyruvate carboxylase gene and enhance or repress its promoter activity, respectively (Yanagisawa 2000, Yanagisawa and Sheen 1998). In *Arabidopsis*, the phloem-specific Dof zinc finger transcription factor, AtDAG1, shows an altered response to R and far-red (FR) light and is involved in seed germination (Papi et al. 2002). Another Dof transcription factor, AtCOG1, is a negative regulator in both the phytochrome A (phyA) and phytochrome B (phyB) signaling pathways. Its expression is light inducible and requires phyA for FR light-induced expression and phyB for R light-induced expression (Park et al. 2003). The *Arabidopsis* OBP3 is a Dof transcription factor, previously named OBF4 Binding Protein 3 (Kang and Singh 2000). It is a component in both phyB and cryptochrome 1 signaling pathways, acting as a positive and negative regulator, respectively (Ward et al. 2005). Furthermore, the Dof transcription factors are also known to regulate flowering time in model plants. In *Arabidopsis*, CYCLING DOF FACTOR 1 (CDF1) negatively regulates the expression of *CONSTANS* (*CO*) which is a key gene in the photoperiodic pathway of flowering time control. Overexpression of *AtCDF1* leads to delayed flowering under long day (LD) conditions in *Arabidopsis* (Imaizumi et al. 2005). Transgenic rice expressing a Dof daily fluctuations 1-antisense (RDD1-antisense (AS)) are smaller and flower later than WT or RDD1-sense rice, and also exhibit shorter grain length (Iwamoto et al. 2009). Transgenic rice overexpressing *OsDof12* (corresponding to *OsRdd4* in Iwamoto et al. 2009) exhibits early flowering under LD conditions.

Circadian clock is internal molecular time-keeping mechanisms that provide organisms with the ability to adjust their growth and physiology and to anticipate diurnal environmental changes such as light and temperature (Yakir et al. 2007a). It has been reported that 6% (453) of genes examined in *Arabidopsis* showed daily fluctuations under the control of circadian clock, including the progress of photosynthesis, stress responses, photoperiodic flowering and sugar metabolism (Harmer et al. 2000). Further investigations found introns and non-coding regions also show circadian rhythms (Hazen et al. 2009).

Of the characterized circadian clock-genes in plants, several encode transcription factors. For example, the zinc transcription factor gene *CO* in *Arabidopsis* (Putterill et al. 1995) and its ortholog *Hd1* in rice (Yano et al. 2000), as well as *AtCDF1* (Imaizumi et al. 2005), *AtCDF2* (Fornara et al. 2009), *OsDof12* (Li et al. 2009) and *Rdd1* (Iwamoto et al. 2009), show circadian rhythms and play important roles in the regulation of flowering time. Moreover, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) that encode Myb-related transcription factors, function in or close to the central oscillator in *Arabidopsis* and exhibit circadian-regulated expression, peaking soon after dawn (Schaffer et al. 1998, Wang and Tobin 1998). In summary, circadian clock-regulated transcription factors induce or repress the expression of genes, causing them to function coordinately at proper points during the day.

Jatropha curcas, a perennial oil plant belonging to the family Euphorbiacea, is widespread in tropical and subtropical areas. It is currently used to prevent erosion and reclaim waste land, and most importantly, its seed has a high oil content that can be processed as biodiesel (Openshaw 2000). However, the prospect of utilizing *J. curcas* for extracting biodiesel is extremely limited by low harvest efficiency because of continuous flowering. It has two flowering peaks during the year in areas of Central America areas, one occurring in May and the other in November. In permanently humid equatorial regions, flowering occurs throughout the year (Heller 1996). In contrast to *Arabidopsis* and rice, little is known about the molecular mechanisms of photoperiodic responses in *J. curcas.* In the present study, a full-length *JcDof1* cDNA was isolated from *J. curcas* seedlings and we investigated its expression patterns under different light conditions.

Materials and methods

Plant material and growth conditions

Mature seeds of *J. curcas* were collected from Panzhihua, Sichuan Province, China. Seeds were planted in pots with 1:1 (v/v) vermiculite with peat medium and incubated at 28 ± 0.5°C for germination. White light (~200 μmol m^{-2} s^{-1}) was provided by cool white fluorescent lights. After exposure to faint light for 3 days to stimulate germination, the pots were transferred to LD (16 h light/8 h dark) or short-day (SD, 8 h light/16 h dark) conditions for 2 weeks at 28 ± 0.5°C. Seedlings grown under continuous dark conditions (DD) for 2 weeks were transferred to continuous light (LL), FR(~60 μmol m^{-2} s^{-1} in 680–700 nm wavelength), R (~80 μmol m^{-2} s^{-1} in 620–630 nm wavelength) or blue (B, ~10 μmol m^{-2} s^{-1} in 450–460 nm wavelength) conditions and cotyledons were sampled. Monochromatic light sources were provided a light emitting diode incubator by Percival Scientific (Perry, IA). Zeitgeber, time-giver, an external oscillator is able to entrain the endogenous clock. *zeitgeber* time (ZT) is used as an objective time based on the phase of the oscillation of the *Zeitgeber* in chronobiology (Rosato and Kyriacou 2006).

DNA-binding activity of JcDof1 proteins in yeast

A recombinant pGADT7-Rec2-*JcDof1* vector containing the entire encoding region of *JcDof1* (GenBank Accession No. GQ256647) from yeast one hybrid (data not shown) and the pHIS2-AAAAG×4 vector were cotransformed into the yeast strain Y187 according to the Clontech protocol (http://www.clontech.com). pGAD-Rec-53/p53HIS2 and pGADT7/pHIS2-AAAAG×4 were used as a positive and negative controls, respectively. The transformed yeast cells were analyzed on synthetic dropout (SD) solid medium lacking Trp, Leu and His and supplemented with 10 m*M* 3-aminotriazole (3-AT) at 30°C for 2–4 days. Subsequently, a colony lift filter assay was applied to monitor the expression of the *LacZ* reporter gene by using X-Gal as a substrate to determine the binding activity of the JcDof1 protein.

Transcriptional activity of JcDof1 proteins in yeast

The coding sequences of *JcDof1* and *JcERF* (JcERF as a typical transcription factor (Tang et al. 2007) and GenBank Accession No. DQ109673) were amplified by polymerase chain reaction (PCR) from the original plasmid using specific primers (*JcDof1:* 5′-GGATCC CCTCAATCAGTGAAGAATC-3′ and 5′-GTCGAC CTCA CGGAACTTGAGTGATCG-3′; *JcERF*: 5′-GAATTC TGA AATGGCTCCAAGAGAGAT-3′ and 5′-GTCGAC GGCT ACTTCAGCCGGAGGAAAG-3′) that contained *Bam*HI (or *Eco*RI) and *Sal*I sites, respectively. They were ligated into the DNA-binding domain pBridge vector and transformed into the yeast strain AH109 according to the Clontech protocol. pBridge-*JcERF* and empty pBridge vector were used as a positive control and negative control, respectively. The transformed yeast cells were analyzed on SD lacking Trp and His at 30°C for 2–4 days. Subsequently, a colony lift filter assay was applied to monitor the expression of the *LacZ* reporter gene by using X-Gal as a substrate to determine the transcription activation of the JcDof1 protein.

Subcellular localization of JcDof1 protein in onion epidermal cells

The *JcDof1* coding sequence was amplified by PCR from the original plasmid using specific primers (*JcDof1*: 5′-GGATCC CTCAATCAGTGAAGAATC-3′ and 5′-ACTAGT CTCATGGAAGTTGATGGATC-3′) that contained *Bam*HI and *Spe*I sites, respectively. The PCR product was ligated to the *GFP* reporter gene in pCAMBIA1302 and sequenced. The fusion construct was then introduced into onion epidermal cells by particle bombardment. The transfected cells were cultured on MS solid medium at 26°C for 24 h and observed under a confocal microscope (Carl Zeiss AIM Version 3.2, Oberkochen, Germany). The *GFP* gene in pCAMBIA1302 was used as a positive control, and both the *JcDof1-GFP* fusion and *GFP* were driven by the 35S promoter.

Reverse transcription PCR (RT-PCR)

Total RNA (1 μg) isolated from roots (R), stem phloem (SP), cotyledon (C) and leaves (L) of 2-week-old *J. curcas* seedlings was used for cDNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI) in a 20-μl reaction volume. The first-strand cDNA mix was used as the template for reverse transcription PCR (RT-PCR). Specific primers for *JcDof1* were 5′-ATC TCCTGCGATGAGTTGC-3′ and 5′-CTCATGGAAGTTG ATGGATC-3′; *ACTIN* primers (GenBank Accession No. GQ256649) were 5′-AGCAACTGGGATGACATGGAG-3′ and 5′-CTACCTTGATCTTCATGCTG-3′. The total volume of the PCR reaction mixture was 20 μl and contained 1 μl cDNA, 0.5 μM of each primer, 1× PCR buffer, 0.4 m*M* dNTP and 1 unit of *Taq* enzyme (Takara). The reaction was incubated at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 45 s at 72°C. Each PCR was repeated at least three times. The amplified fragments were separated on a 1% w/v agarose gel and stained with ethidium bromide. The agarose gel was scanned using a UV2000 imaging system (AlphaImager, San Leandro, CA). A *J. curcas ACTIN* gene was amplified as a control in these experiments with 29 cycles.

Quantitative Real-Time PCR (Q-PCR)

Two-week-old cotyledons were sampled every 3 h under different light conditions and DNaseI-treated total RNA (1 μg) was extracted and used for cDNA synthesis with a M-MLV reverse transcriptase (Promega, Madison, WI) in a 20-μl reaction volume. The specific primers to amplify *JcDof1* were 5′-TCTGATCCTCCGGCTATC TCCTG-3′ and 5′-GGGTGTTTTTGGGTTCTCATTCG-3′; primers used to amplify the light-harvesting chlorophyll a/b-binding protein gene (*CAB*, GenBank Accession No. GQ984214) were 5′-GTGATTATGGGTGGGATA CTGCT-3′ and 5′-GCCTTGAACCAAACTGCCTCT-3′; and primers used to amplify *ACTIN* were 5′-CACCATC ACCAGAATCCAGCAC-3′ and 5′-TCCACTAAGCCCTA AAGCCAAC-3′. The 20-μl real-time PCR reaction was composed of the following: 0.2 μ*M* of each primer, 1 μl of 10-fold diluted cDNA (equivalent to approximately 0.05 ng of mRNA); 10 μl of SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). The synthesized cDNAs were amplified by using the Stratagene

MX3000P sequence detection system and associated program (2 min at 95°C, and 40 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 15 s; 1 cycle of 95°C for 10 s, 56°C for 15 s, and 95°C for 30 s). The comparative threshold cycle (CT) method was used to determine the relative degree of gene expression, with the expression of *ACTIN* used as an internal control. Mean values of $2^{-\Delta CT}$ ($\Delta C_T = C_{T,\ \text{gene of interest}} - C_{T,\ ACTIN}$) were calculated from three independent experiments.

Results

Isolation and characterization of *JcDof1* cDNA

The full-length *JcDof1* cDNA, isolated from *J. curcas* seedlings by a yeast one hybrid library (data do not show), was 1825 bp and included 5′ and 3′ flanking regions of 101 and 308 bp, respectively (Fig. 1). *JcDof1* contained an open reading frame of 1416 bp encoding a putative protein of 471 amino acid residues with a predicted molecular mass of 51.41 kDa and a predicted isoelectric point of 7.05. A comparison of the genomic DNA sequence to the isolated cDNA indicated that *JcDof1* had a 1090-bp intron upstream of the Dof domain-coding region. JcDof1 had a zinc finger motif (CX2CX21CX2C, X stands for any amino acid) a characteristic of the plant-specific Dof transcription factors (Yanagisawa and Sheen 1998). Alignment of the deduced amino acid sequence of JcDof1 with other Dof homolgs showed that JcDof1 shared four highly conserved domains. One domain, the zinc finger motif, is located in the N-terminal region and is known to mediate DNA binding or protein-protein interactions (Yanagisawa 2002), whereas the other conserved domains are located in the C-terminal transcriptional activation regions (Fig. 1A, B). The full-length cDNA sequence of *JcDof1* has been deposited in the NCBI GenBank under Accession No. GQ256647.

A phylogenetic tree was constructed from the Dof proteins (Fig. 1C) and those characterized previously. These Dof proteins have all been reported to be involved in the plant response to light, except for Ascorbate Oxidase gene Binding Protein (CmAOBP) and AtOBP1, AtOBP2. Compared with the phylogenetic tree reported by Lijavetzky (2003), we found that JcDof1 clustered in group D with *Arabidopsis* CDF1-3 (Imaizumi et al. 2005) and COG1 (Papi et al. 2002, Park et al. 2003), *Oryza sativa* Rdd1-5 (Iwamoto et al. 2009) and *Cucurbita maxima* AOBP (Kisu et al. 1998). Among these characterized Dof genes, *AtCDFs* and *OsRdds* have been reported as circadian-regulated genes (Fornara et al. 2009, Imaizumi et al. 2005, Iwamoto et al. 2009, Li et al. 2009).

JcDof1 encodes a putative Dof-like transcription factor

A yeast one-hybrid assay was employed to test the DNA-binding activity of JcDof1. Recombinant pGADT7-Rec2-*JcDof1* and pHIS2-AAAAG×4 were cotransformed into the yeast strain Y187 harboring the reporter gene *LacZ*. Transformed yeast cells grew on SD solid medium lacking Trp, Leu and His and supplemented with 10 m*M* 3-AT at 30°C after 3 days. Similar results were obtained using positive control constructs (pGAD-Rec2-53 plus p53HIS2), whereas the negative control (pGADT7 plus pHIS2-AAAAG×4) did not grow on the identical medium. In β-galactosidase activity assays, yeast cells that could grow on the same SD medium turned blue according to colony lift filter assays (Fig. 2A), verifying that JcDof1 could specifically bind the AAAAG element.

To investigate the transcriptional activation activity of JcDof1, the recombinant plasmid pBridge-*JcDof1*, pBridge-*JcERF* (JcERF as a typical transcription factor (Tang et al. 2007) and a positive control) and empty pBridge (as a negative control) were transformed into the yeast strain AH109 (Clontech) harboring the *His* and *LacZ* reporter genes. Yeast transformed with pBridge-*JcDof1*, pBridge-*JcERF* plasmids grew well on SD medium lacking Trp and His and also activated the *LacZ* reporter in colony lift assays. In contrast, yeast transformed with the negative control plasmid did not grow in the same medium (Fig. 2B). These results indicated that JcDof1 functioned as a transcriptional activator in yeast.

To examine the nuclear localization of JcDof1, a plant expression vector in which *JcDof1* was fused to *GFP*, pCAMBIA-1302-*JcDof1*, as well as a GFP expression vector, pCAMBIA-1302, were used to transform onion epidermal cells by particle bombardment, respectively. Protein expression was observed under a laser scanning confocal microscope. The JcDof1-GFP fusion proteins were confined to the cell nucleus, whereas control GFP protein was observed in the cytoplasm and the cell nucleus (Fig. 2C). Although GFP is localized to the cytosol, its molecular weight is well below the exclusion limit of nuclear pores and is therefore often observed diffusing into the plant cell nucleus (ref). These results indicated that JcDof1 was localized to the nucleus, and together with yeast one-hybrid data, confirmed that JcDof1 was a typical member of the Dof transcription factors.

RT-PCR was performed to investigate the expression of *JcDof1* in the root (R), SP, cotyledon (C) and leaves (L) of 2-week-old *J. curcas* seedlings at ZT4 under LD conditions. The spatial pattern of *JcDof1* expression showed that *JcDof1* was expressed in all organs tested, but was markedly lower in roots (Fig. 3).

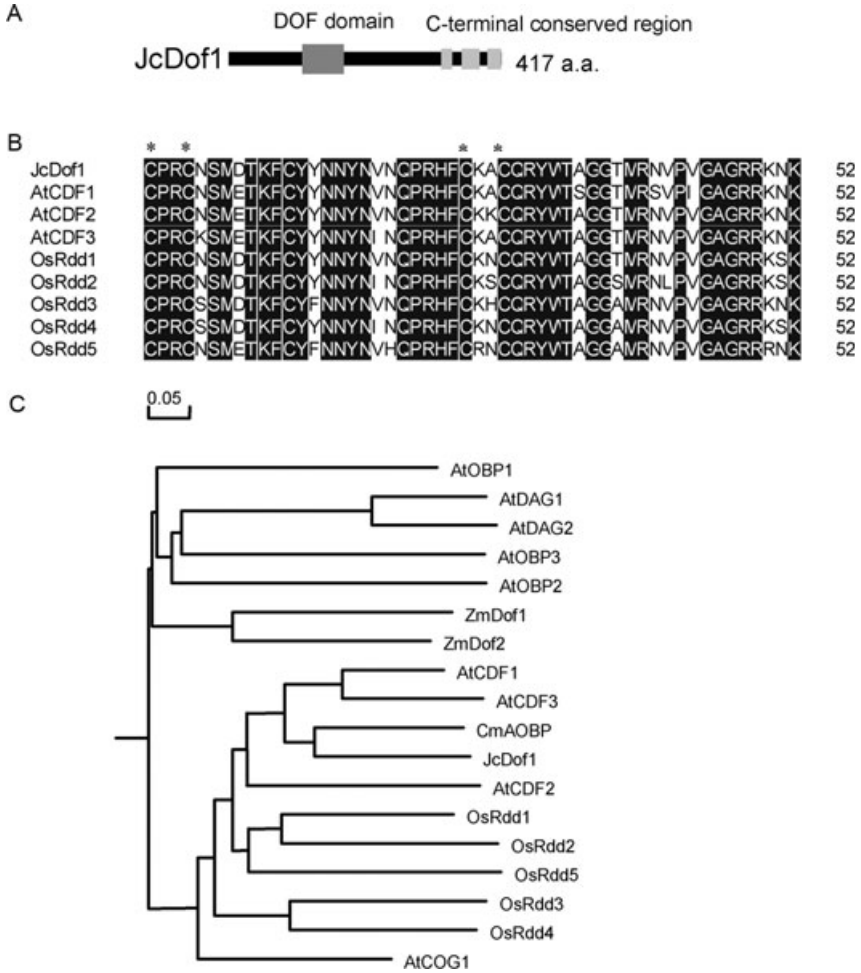

Fig. 1. Structure and phylogenetic relationships among Dof proteins. (A) Schematic drawing of the Dof domain (shown as a dark grey bar) and C-terminal conserved region (shown as light grey bars) in the JcDof1 protein. (B) Alignment of the Dof domain in JcDof1 to those in other Dof proteins. Identical amino acid residues are boxed in black. Four conserved cysteine residues in the Dof domain are indicated by asterisks. (C) Phylogenetic tree of JcDof1 and other Dof proteins. The scale bar corresponds to 0.05 estimated amino acid substitutions per site. At, *Arabidopsis thaliana*; Cm, *Cucurbita maxima*; Jc, *Jatropha curcas*; Os, *Oryza sativa*; Zm, *Zea mays*. Accession numbers or gene codes corresponding to the Dof proteins are as follows: AtCDF1-3 (At5g62430, At5g39660, At3g47500); AtCOG1 (At1g29160); AtDAG1 and AtDAG2 (At3g61850, At2g46590); AtOBP1-3 (At3g50410, At1g07640, At3g55370); CmAOBP (D45066); JcDof1 (GQ256647); OsRdd1-5 (AP0001383, AY224430, AE017092, AY224429, AP000836), ZmDof1-2 (X66076, X79934).

Expression profiling of *JcDof1* under different photoperiods

To investigate *JcDof1* expression in response to photoperiod, 2-week-old *J. curcas* seedlings grown under LD conditions or short day (SD) conditions were harvested every 3 h, and their transcript levels were monitored by Quantitative real-time PCR (Q-PCR) assay. As indicated in Fig. 4A (Supporting information Fig. S1 illustrates a comparable semi-quantitative RT-PCR assay) under LD, the expression levels of *JcDof1* were constitutively low from ZT1 to ZT16, diminished from ZT16 to ZT19 and then increased rapidly from ZT19 to ZT22. Under SD conditions, *JcDof1* expression levels were constitutively low from ZT1 to ZT7, diminished from ZT7 to ZT16 and then rapidly increased from ZT16 to ZT22. Although the oscillating curves of *JcDof1* transcripts under LD and SD were not identical, it was evident that their oscillating patterns were similar. That is, under either photoperiod, *JcDof1* transcripts gradually decreased before dusk and were retained at a very low level during the night, but increased significantly before dawn. These results indicated that the expression of *JcDof1* under LD or SD exhibited the circadian oscillations that accurately depend on different day lengths corresponding to seasonal changes.

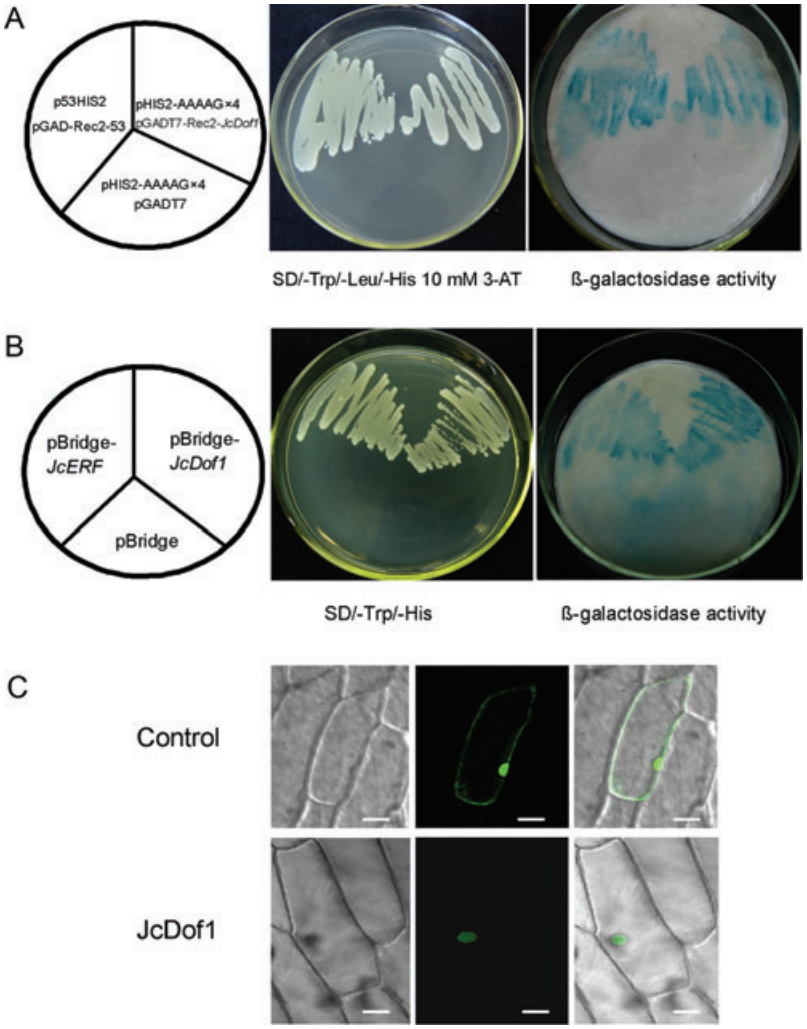

Fig. 2. Transcriptional binding activity, transcriptional activation activity and nuclear localization of JcDof1 protein. (A) Transcriptional binding activity of JcDof1. The left panel illustrates the position of each transformed yeast cell. The middle panel illustrates transformed yeast cells growing on SD/-Trp/-Leu/-His plus 10 m*M* 3-AT at 30°C and the right panel illustrates β-galactosidase activity. Yeast Y187 cells transformed with p53HIS2 and pGAD-Rec2-53 vectors were used as a positive control, whereas those transformed with pHIS2-AAAAG×4 and pGADT7 vectors were a negative control. (B) Transactivation activation activity of the JcDof1 protein. The left panel illustrates the position of each transformed yeast cell, the middle panel illustrates growth of yeast cells on SD/-Trp/-His, the right panel is β-galactosidase activity. Yeast AH109 cells transfected with pBridge-*JcERF* and pBridge vector were used as the positive and negative controls, respectively. (C) Subcellular localization of the JcDof1-GFP fusion protein in onion epidermal cells. GFP alone (control) or JcDof1-GFP fusion proteins were expressed transiently under the control of the CaMV 35S promoter in onion epidermal cells. Images are bright field (left) for the morphology of the cell, dark field (middle) for green fluorescence, and merged (right). Bars are 50 μm.

Expression profiling of *JcDof1* under light/dark conditions

To determine *JcDof1* expression and *JcCAB* in response to light, 2-week-old etiolated *J. curcas* seedlings were harvested every 3 h for 5 days (the 1st day under DD, from the 2nd day to the 4th day under LL, the 5th day under DD again). Transcription of *JcDof1* and *JcCAB* were monitored by Q-PCR assay. As indicated in Fig. 4B, during the 1st day when seedlings were under DD, expression of *JcDof1* remained at basal levels without an obvious oscillatory pattern. During this period some abnormal phenotypes, such as elongated and curled hypocotyls, etiolated cotyledons, and undeveloped leaves were observed, which were different from seedlings maintained under LD/SD (Fig. S2). However, after the seedlings were exposed to LL, oscillating curves were observed in the first 24 h (Fig. 4B) and continued to the 4th day. Maximal expression levels of *JcDof1* under

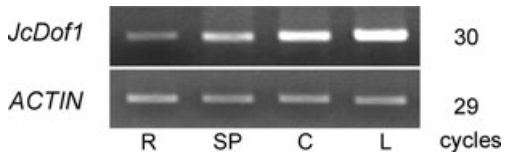

Fig. 3. Expression of the *JcDof1* gene at ZT4 in seedling tissues under LD conditions. Total RNA isolated from roots (R), stem phloem (SP), cotyledon (C), leaves (L) were used in semi-quantitative RT-PCR. PCR products were separated by electrophoresis in 1.0% (w/v) agarose gels and visualized with ethidium bromide. Expression of an *ACTIN* gene was used as an internal control.

LL were two-fold higher than levels under DD, and they were about 10-fold higher than the minimal levels in each cycle. Over the course of 3 days of LL, the etiolated seedlings gradually turned green and grew more robust. Interestingly, in the 5th day when the de-etiolated seedlings showing *JcDof1* expression oscillation were shifted to DD, oscillation in *JcDof1* transcription was retained. As a control, transcription of *chlorophyll a/b-binding protein* (*JcCAB*), a light-regulated gene (67% amino acid identity between *JcCAB* and *AtCAB2*), was barely detectable under DD conditions (the 1st day) and was significantly up-regulated and maintained daily oscillation under LL (from 2nd day to 4th day) until under DD again (the 5th day) (Fig. 4B). These results indicated that light is important to initiate circadian expression of *JcDof1*.

To further analyze the degree of *JcDof1* oscillation dependence on light, one cotyledon of each 2-week-old *J. curcas* seedling grown under DD conditions was wrapped in tinfoil to avoid light, and seedlings were then transferred to light conditions. Both the wrapped and the induced cotyledons were harvested at ZT0, 1, 3, 6, 9 and 12 after exposure to light. During this period of time (Fig. 4C), the degree of *JcDof1* transcription in the wrapped cotyledons remained at basal levels as those under DD, whereas of the lighted cotyledons gradually decreased as previously observed for those exposed to LD/SD/LL conditions. These data demonstrated that light accurately mediated the daily oscillation of *JcDof1* transcription.

Expression profiling of *JcDof1* under FR/R/B light

To test if the regulation of *JcDof1* and *JcCAB* expression was dependent on light quality (wave-length), 2-week-old *J. curcas* etiolated seedlings were transferred to FR, R and B light conditions, respectively. The cotyledons were harvested at ZT0, 1, 3, 6, 9 and 12 (Fig. 4D). Under R and B light conditions, *JcDof1* transcripts gradually decreased to low levels as previously observed for white light conditions, but this decrease was not observed under FR conditions. As a control, *JcCAB* transcripts were up-regulated under R, FR and B light conditions (Fig. 4D). It was, therefore, possible that different light quality might entrain different genes, with R and B light downregulating the *JcDof1* expression and FR without reaction.

Discussion

Transcription factors in plants regulate gene expression and/or mediate signal transduction events. The Dof family of transcription factors is involved in diverse plant-specific biological processes (Yanagisawa 2004). In this study, the isolated *JcDof1* coding sequence consisted of two major domains: an N-terminal conserved DNA-binding domain and a C-terminal domain involved in transcriptional regulation (Fig. 1A). Moreover, *JcDof1* containing only one intron upstream of the Dof domain-coding region region shared higher sequence similarity with *AtCDF1-3* than with *OsRdd1-5* containing noughty/few introns, which is likely because of the fact that *J. curcas* and *Arabidopsis* are both dicots. Yeast one hybrid assays and subcellular localization to the nucleus confirmed that *JcDof1* was a typical member of the Dof transcription factor family (Fig. 2A–C). The spatial pattern of *JcDof1* expression at ZT4 in the cotyledon, leaf, and SP overlapped with that of *AtCDF1,* except in the root. Though Imaizumi (2005) implied that no CDF1::GUS activity was detected in roots under LD/SD conditions, faint expression of *JcDof1* was observed in *J. curcas* roots.

Most organisms adjust their physiology and metabolism in synchronization with the diurnal and seasonal time by using an endogenous circadian system. In plants, light and temperature signals interact with the circadian system to regulate the circadian rhythmicity of physiological and developmental processes so that they occur at optimal times (Bieniawska et al. 2008, Edwards et al. 2006, Yakir et al. 2007a). Our data demonstrated that the daily oscillations in *JcDof1* transcription occurring in LL/SD/LD conditions (Fig. 4A, B) were similar to those observed for *AtCDF1*, *OsRdd1* and *OsDof12* (Imaizumi et al. 2005, Iwamoto et al. 2009, Li et al. 2009).This data correlates with our multiple alignment and phylogenetic tree (Fig. 1B, C). Moreover, like *LATE ELONGATED HYPOCOTYL* (*LHY*) and *TIMING OF CAB EXPRESSION1* (*TOC1*) genes constituting the central oscillator in *Arabidopsis* (Kikis et al. 2005), *JcDof1* transcripts remained at basal levels, without detectable oscillating curves, in etiolated cotyledons (Fig. 4B) and in wrapped cotyledons shielded from light (Fig. 4C), whereas oscillating curves were rapidly induced under light conditions (Fig. 4B). In

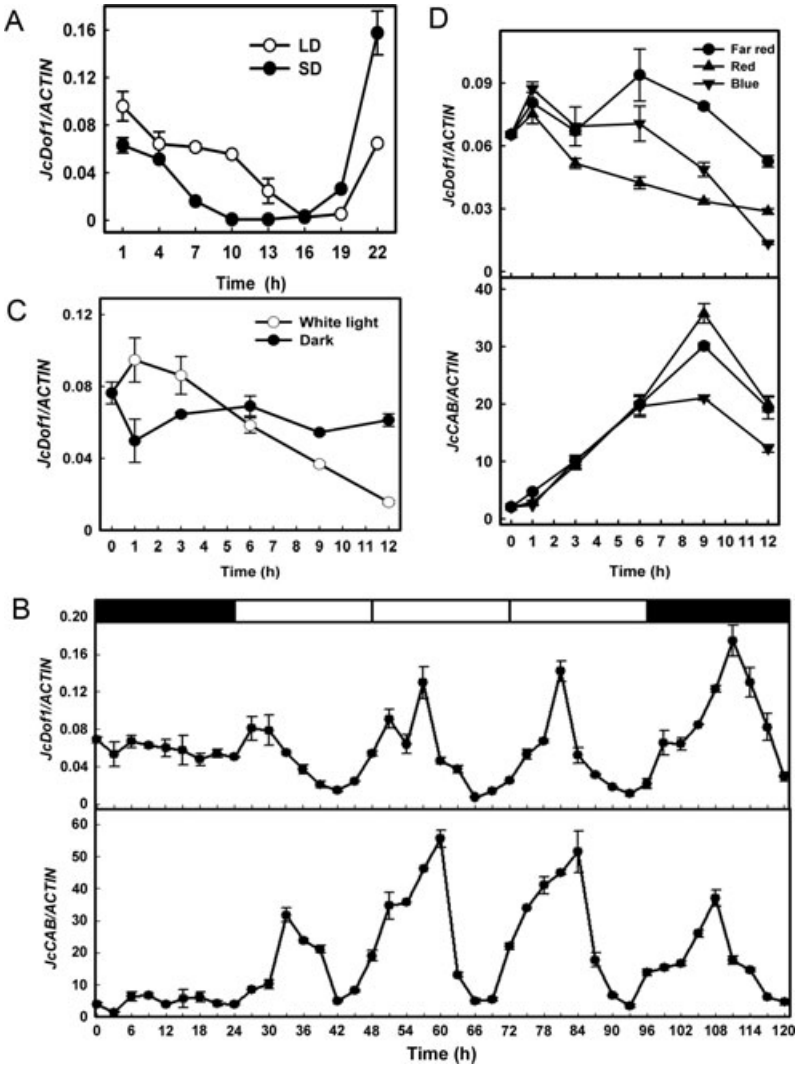

Fig. 4. Q-PCR analysis of *JcDof1* expression under different light conditions. (A) Two-week-old *J. curcas* seedlings under long day (LD, 16 h light/8 h dark) or short day (SD, 8 h light/16 h dark). Leaves were harvested at 3-h intervals for a total of 24 h. (B) Two-week-old *J. curcas* seedlings under DD (for 24 h) were transferred to LL (for 72 h) and then DD (for 24 h). Dark and light periods are indicated by black and white bars, respectively. The cotyledons were harvested at 3 h intervals for a total of 120 h. (C) One cotyledon of each 2-week-old *J. curcas* seedling grown under DD conditions was wrapped in tinfoil to avoid light, then seedlings were transferred to LL. Two cotyledons were harvested at ZT0, 1, 3, 6, 9 and 12, respectively. (D) Two-week-old *J. curcas* seedlings under DD were transferred to FR, R or B light. The cotyledons were harvested at ZT0, 1, 3, 6, 9 and 12. Expression of an *ACTIN* gene was used as an internal control. All *JcDof1* and *JcCAB* transcript levels are shown as a relative value to the internal control. Data are means ± SEM from three independent experiments.

addition, the rhythmic oscillation of *JcDof1* expression was retained after de-etiolated seedlings were returned to DD at the 5th day (Fig. 4B). In rice, *OsRdd1*, *OsLHY* and *OsTOC1* gene expression also showed arrhythmic accumulation patterns in etiolated coleoptiles (Iwamoto et al. 2009). These results suggested that the basal or arrhythmic expression of oscillator genes in etiolated seedlings or DD conditions is a common phenomenon and *JcDof1* was a light-mediated circadian-clock gene. Based on the function of AtCDF1, OsRdd1 and OsDof12 in the regulation of flowering time, we presumed *JcDof1* must play very crucial roles in the 'photoperiodic' pathway responding to *J. curcas* flowering. In some areas, the main harvest of *J. curcas* occurs over the course of several months because of continuous flowering, which leads to low harvest efficiency. Therefore, the development of transgenic plants may not only lead to a better understanding of *JcDof1* function, but also to regulate the flowering time of this economically important species.

Plants perceive subtle changes in light quality through a set of photoreceptors, including the R/FR absorbing phytochromes, the B/UV-A absorbing cryptochromes and phototropins. The phytochromes and cryptochromes have both unique and redundant roles in mediating photomorphogenic responses, including hypocotyl elongation, cotyledon/leaf expansion, flowering time and the circadian clock (Lin and Shalitin 2003, Neff et al. 2000). By comparing the expression levels of *JcDof1* under FR, R and B light conditions, we found that *JcDof1* expression was sensitive to R/B light and the decrease in expression was similar to that observed under white light, but *JcDof1* expression was insensitive to FR light (Fig. 4D). Similar patterns were observed for *CCA1*, an oscillator gene in *Arabidopsis. CCA1* transcripts were relatively stable in the dark and in FR light, but had a short half-life under R and B light (Yakir et al. 2007b). On the other hand, Iwamoto (2009) showed that *Rdd1* is a circadian clock and phy-regulated gene. It responded to FR in wild-type plants, phyB- or phyC- deficient mutants, but not in phyA-deficient mutants, although R light-responsive *Rdd1* expression was detected in wild-type plants, phyA- or phyB-deficient mutants. In contrast, *JcCAB* expression was abundant under all three light conditions. These results suggested that phytochrome and cryptochrome pathways are likely to mediate the circadian oscillation of *JcDof1* expression in *J. curcas*.

In addition, at a cutoff of 50% amino acid identity, orthologous proteins of JcDof1 were found in a wide spectrum of organisms (HomoloGene:55930 in the National Center for Biotechnology Information database; http://www.ncbi.nlm.nih.gov/sites/Entrez?db=homologene). These organisms include *Helianthus petiolaris* (ACJ13919.1), *Medicago truncatula* (ACJ85660.1), *Populus trichocarpa* (XP_002323863.1); *Rinus communis* (EEF30530.1), *Vitis vinifera* (XP_002269461.1) (Fig. S3). The prevalence of JcDof1 orthologs in a wide spectrum of organisms implies a general involvement of these proteins in growth and/or developmental processes. Reports of biological functions for most of these proteins remain limited. Our studies have offered us increased understanding of the expression patterns of *JcDof1* in response to light signals. Further work is required to clarify the mechanism of the complex photoperiodism in *J. curcas* and the functional elucidation of these orthologous proteins in economically important plants. In particular, we aim to discover how *J. curcas* senses when to flower and manipulate the regulation of flowering time to increase the economic potential of this species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. RT-PCR analysis of *JcDof1* expression under long day (LD, 16 h light/8 h dark), short day (SD, 8 h

dark/16 h light). The leaves of 2-week-old *J. curcas* seedlings were harvested at 3 h intervals for a total of 24 h. Gene-specific primers amplifying *JcDof1* and *ACTIN* genes were used (Fig. 3). PCR products were separated by electrophoresis in 1.0% (w/v) agarose gels and visualized with ethidium bromide. Expression of an *ACTIN* gene was used as an internal control. Dark and light periods are indicated by black and white bars, respectively.

Figure S2. Two-week-old seedlings in DD/LD. Bars are 1 cm.

Figure S3. Phylogenetic tree of JcDof1 and other orthologous proteins. The scale bar corresponds to 0.05 estimated amino acid substitutions per site. At, *Arabidopsis thaliana*; Cm, *Cucurbita maxima*; Jc, *Jatropha curcas*; Os, *Oryza sativa*; Zm, *Zea mays*. Accession numbers or gene codes corresponding to the Dof proteins are as follows: AtCDF1-3 (At5g62430, At5g39660, At3g47500); AtCOG1 (At1g29160); AtDAG1 and AtDAG2 (At3g61850, At2g46590); AtOBP1-3 (At3g50410, At1g07640, At3g55370); CmAOBP (D45066); JcDof1 (GQ256647); OsRdd1-5 (AP0001383, AY224430, AE017092, AY224429, AP000836); ZmDof1-2 (X66076, X79934); ACJ13919.1 (*Helianthus petiolaris*); ACJ85660.1 (*Medicago truncatula*); CAN60326.1(*Vitis vinifera*); EEF30530.1 (*Rinus communis*); XP_002323863.1 (*Populus trichocarpa*).

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