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Original Paper

Diurnal expression of *CONSTANS-like* genes is independent of the function of cycling DOF factor (CDF)-like transcriptional repressors in *Physcomitrella patens*

Tetsuya Ishida[1], Takumi Sugiyama[2], Nobumitsu Tabei[2], Shuichi Yanagisawa[1,2,*]

[1] Biotechnology Research Center, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan; [2] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
* E-mail: asyanagi@mail.ecc.u-tokyo.ac.jp Tel: +81–3–5841–3066 Fax: +81–3–5841–8030

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Abstract The Dof-type transcriptional repressors CYCLING DOF FACTORS (CDFs) directly suppress the expression of *CONSTANS* (*CO*), which encodes a key regulator of photoperiodic gene expression, day-length perception and the floral transition in *Arabidopsis thaliana*. The genes encoding CDF-like (PpDof3 and PpDof4) and CO-like (PpCOL1-PpCOL3) proteins are present in the genome of the moss *Physcomitrella patens*, although *P. patens* lacks the genes encoding homologues of FLAVIN-BINDING KELCH REPEAT F-BOX1 (FKF1) and GIGANTEA (GI), which control the stability of CDFs in *A. thaliana*. In the current study, we investigated whether the functions of PpDof3 and PpDof4 are associated with the expression of *PpCOL1-PpCOL3* in *P. patens*. We found that the diurnal expression patterns of *PpDof3* and *PpDof4* are similar to those of *CDF* genes and that like CDF1 from *A. thaliana*, PpDof3 and PpDof4 function as transcriptional repressors. However, targeted disruptions of *PpDof3* and *PpDof4* did not affect the expression of *PpCOL1-PpCOL3*, indicating that the expression of *COL*s is independent of the functions of PpDof3 and PpDof4 in *P. patens*.

Key words: *CONSTANS-like* gene, cycling DOF factor, *Physcomitrella patens*, targeted gene disruption.

Dof transcription factors, which possess plant-specific Dof DNA-binding domains, are present in both vascular and non-vascular plants (Moreno-Risueno et al. 2007; Shigyo et al. 2007) and play roles in a variety of biological processes in the plant kingdom (Sugiyama et al. 2012; Yanagisawa 2002). Recently, a Dof transcription factor in *Arabidopsis thaliana* (Arabidopsis) was identified as a transcriptional repressor of *CONSTANS* (*CO*), which encodes a master regulator of photoperiodic gene expression and the floral transition from the vegetative phase to the reproductive phase (Imaizumi and Kay 2006; Samach and Coupland 2000; Song et al. 2010; Valverde 2011); this factor was designated CYCLING DOF FACTOR 1 (CDF1). Subsequently, four Dof proteins in Arabidopsis, CDF2, CDF3, CDF4 and CDF5, were identified as CDF-related factors (Fornara et al. 2009; Imaizumi et al. 2005). All CDFs except CDF4 play redundant roles in repressing *CO* expression and photoperiodic flowering in Arabidopsis (Fornara et al. 2009). Consistent with the hypothesis that in general, closely related Dof transcription factors share small amino acid motifs outside the highly conserved Dof DNA-binding domain (Lijavetzky et al. 2003; Moreno-Risueno et al. 2007; Shigyo et al. 2007; Yanagisawa 2002), all CDFs except CDF4 have conserved amino acid motifs in their C-terminal regions (Fornara et al. 2009; Imaizumi et al. 2005). Thus, these conserved amino acid motifs are characteristic of CDFs.

Since *CO* expression is controlled by both light signaling and circadian clocks, and since CO protein functions as a transcriptional regulator of the expression of *FLOWERING LOCUS T* (*FT*), whose product is a mobile signal from the phloem to the meristem, CO integrates information on day length in leaves and regulates the floral transition in meristems in response to seasonal changes (Samach and Coupland 2000; Imaizumi and Kay 2006; Song et al. 2010; Valverde 2011). CDF-mediated transcriptional regulation of the photoperiodic expression of *CO* as well as post-translational regulation of the stability of CO protein (Jang et al. 2008; Valverde et al. 2004) are key regulatory mechanisms in the CO-mediated regulatory pathway for photoperiodic gene expression and the floral transition in Arabidopsis (Song et al. 2010; Valverde 2011). CDFs interact

Abbreviations: Arabidopsis, *Arabidopsis thaliana*; CDF, CYCLING DOF FACTOR; CO, CONSTANS; COL, CONSTANS-LIKE; FKF1, FLAVIN-BINDING KELCH REPEAT F-BOX1; FT, FLOWERING LOCUS T; GI, GIGANTEA.

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with FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1) and GIGANTEA (GI) (Imaizumi et al. 2005; Sawa et al. 2007), which were identified as core components of the CO-mediated regulatory pathway based on the phenotypes of their mutants (Fowler et al. 1999; Imaizumi et al. 2003; Sawa et al. 2007). Furthermore, FKF1, which interacts with GI nuclear protein, controls the stability of CDFs (Imaizumi et al. 2005; Sawa et al. 2007). Thus, CDFs are downstream components of FKF1 and GI in the CO-mediated regulatory pathway in Arabidopsis.

The floral transition is a developmental process unique to flowering plants, while effects of photoperiod on the growth phase transition was found in gymnosperms, ferns and bryophytes as well. In agreement with this fact, *CO-like* genes (*COLs*) have been discovered in the moss *Physcomitrella patens* and the green alga *Chlamydomonas reinhardtii* as well as flowering plants (Valverde 2011). *C. reinhardtii* contains the *COL* gene *CrCO*, which plays a pivotal role in photoperiod-regulated control of growth and metabolism in *C. reinhardtii* and can complement the *co* mutation in Arabidopsis (Serrano et al. 2009). On the other hand, in the genome of *P. patens*, a model plant studied to gain insights into the evolution of specific gene regulatory networks in plants (Quatrano et al. 2007), three *COL* genes (*PpCOL1-PpCOL3*) have been identified (Shimizu et al. 2004; Zobell et al. 2005). *PpCOLs* are more closely related to Arabidopsis *CO-like* genes (*AtCOL3-AtCOL5*) rather than *CO* itself (Zobell et al. 2005).

We previously reported that two Dof proteins from *P. patens*, PpDof3 and PpDof4, are probably CDF homologues, because they possess amino acid motifs characteristic of CDFs (Sugiyama et al. 2012). In spite of the presence of the genes encoding COL and CDF-like proteins in *P. patens*, a recent comprehensive analysis of the *P. patens* genome revealed that this moss lacks homologues of FKF1 and GI (Holm et al. 2010). Thus, in the current study, we characterized the roles of PpDof3 and PpDof4 as transcription factors and investigated the relationship between the functions of PpDof3 and PpDof4 and the expression of *PpCOL1-PpCOL3* using gene targeting.

Materials and methods

Biological materials and growth conditions

P. patens (Hedw.) Bruch & Schimp. ssp. patens Tan (Ashton and Cove 1977) was used in this study. Protonema of wild-type and transformed *P. patens* were grown on agar plates containing BCDATG medium (1 mM $MgSO_4$, 1.84 mM KH_2PO_4, 10 mM KNO_3, 45 μM $FeSO_4$, 0.22 μM $CuSO_4$, 10 μM H_3BO_3, 0.23 μM $CoCl_2$, 0.1 μM Na_2MoO_4, 0.19 μM $ZnSO_4$, 2 μM $MnCl_2$, 0.17 μM KI, 1 mM $CaCl_2$, 5 mM ammonium tartrate and 0.5% glucose) at 25°C under a day/night cycle of 16/8 h with approximately 50 μmol photons m^{-2} s^{-1} light. To analyze gene expression, protonema of wild-type and *ppdof* disruptant lines were regenerated from single protoplasts and grown for 2 weeks on agar plates containing modified BCDATG medium (made with 10 mM CaCl and 6% mannitol rather than 1 mM CaCl and 0.5% glucose), followed by transfer to agar plates containing BCDATG medium. After 10 days of cultivation on agar plates, protonema samples were sampled every 4 h for 28 h.

Generation of *P. patens* disruptants and genomic Southern blot analysis

To construct gene-targeting vectors, genomic DNA fragments containing *PpDof3* or *PpDof4* were amplified by PCR using gene-specific primers (Supplemental Table S1) and cloned into pGEM-T (Promega, Madison, WI, USA). Then, the regions encoding the Dof domains of PpDof3 and PpDof4 in the resultant plasmids were replaced with a 35S promoter-driven hygromycin resistance gene or a 35S promoter-driven kanamycin resistance gene (Sugiyama et al. 2012). Transformation of *P. patens* with these targeting vectors was performed by the polyethylene glycol method, and the transformants were selected on agar plates containing appropriate concentrations of antibiotics according to the method described previously (Sugiyama et al. 2012). Genomic Southern blot analysis was performed with *Eco*RV-digested genomic DNA from the protonema of the wild type and disruptant lines, as described previously (Sugiyama et al. 2012). PpDof3- and PpDof4-specific DNA probes were prepared by PCR using the gene-specific primers listed in Supplemental Table S1.

Quantitative RT-PCR (qPCR)

Total RNA was prepared from the protonema samples using a Fruit-mate for RNA Purification kit (Takara Bio, Shiga, Japan) and Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription reactions were performed with oligo$(dT)_{15}$ primers or random hexamer primers using SuperScript® II reverse transcriptase (Life Technologies Japan, Tokyo, Japan). Quantitative and semi-quantitative PCR were performed as described previously (Sugiyama et al. 2012). The primers used are listed in Supplemental Table S1.

Protoplasts transient assays

For transient assays using maize mesophyll protoplasts, a reporter plasmid containing the luciferase reporter gene (*LUC*), under the control of a LexA-binding site-containing promoter in which eight copies of LexA-binding sites were placed between the 35S enhancer and the 35S minimal promoter, was utilized (Sugiyama et al. 2012). For construction of effector plasmids, the plant expression vector for LexA-VP16 fusion protein was utilized (Yanagisawa et al. 2003). The DNA sequence encoding the VP16 transcriptional activation domain in the vector was replaced with the entire coding sequence for PpDof3 or PpDof4, which were obtained by PCR using gene-specific primers (Supplemental Table S1). All plasmids

constructed were verified by DNA sequencing. Co-transfection of reporter and effector plasmids into protoplasts was performed using an internal control plasmid containing *uidA* [*β*-glucuronidase (GUS) gene] under the control of the maize ubiquitin promoter, as described previously (Yanagisawa 2000). LUC and GUS activity were measured as described previously (Konishi and Yanagisawa 2008).

Results

Diurnal expression of PpDof3 *and* PpDof4

In a previous phylogenetic analysis of the Dof transcription factor family, all CDFs formed a clade together with two other Arabidopsis Dof proteins [the uncharacterized Dof protein AtDof1.3 and COG1, which is involved in phytochrome signaling (Park et al. 2003)]. This clade also contains six Dof proteins from *P. patens* (PpDof1 to PpDof6) and a Dof protein that is uniquely present in *C. reinhardtii* (Shigyo et al. 2007). Furthermore, by isolation of full-length cDNAs encoding PpDof1–PpDof6, it was revealed that among Dof proteins from *P. patens*, only PpDof3 and PpDof4 possess conserved amino acid motifs that characterize CDFs (Sugiyama et al. 2012). PpDof3 and PpDof4 do not possess all three motifs characterizing CDFs, but they have two motifs (Figure 1). Thus, PpDof3 and PpDof4 have closest relationships with CDFs. We therefore investigated the expression patterns of *PpDof3* and *PpDof4* under long days (LDs) and short days (SDs), together with *PpDof1*, *2*, *5* and *6*. As shown in Figure 2, *PpDof3* and *PpDof4* exhibited similar diurnal expression patterns, while *PpDof1*, *2*, *5* and *6* did not obviously show any diurnal expression pattern. The expression levels of *PpDof3* and *PpDof4* were highest at the beginning of the light period and subsequently decreased under both LDs

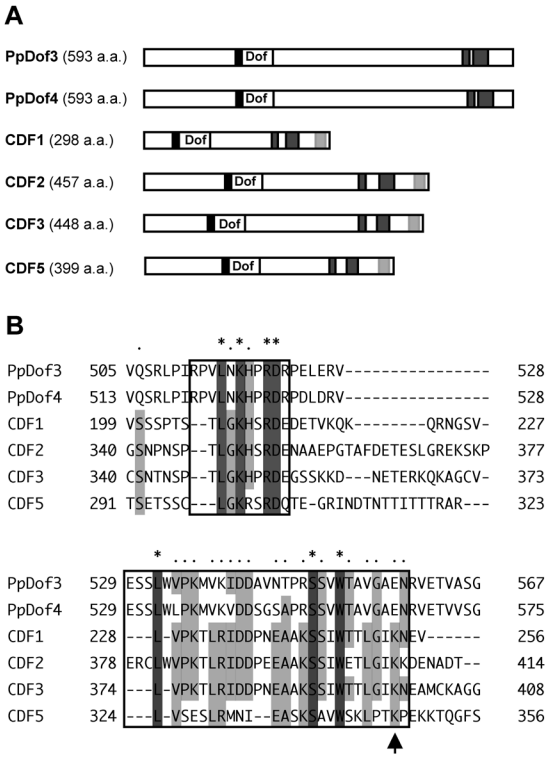

Figure 1. Structures of PpDof3 and PpDof4 from *P. patens* and CDF1–CDF3 and CDF5 from Arabidopsis. (A) Domain structures. The Dof domain is indicated. A small conserved motif flanking the Dof domain and two motifs conserved in the C-terminal regions of PpDof3, PpDof4, CDF1–CDF3 and CDF5 are also indicated by black and dark grey boxes, respectively. Light grey boxes indicate an additional motif that is conserved in CDFs but not found in PpDof3 or PpDof4. (B) Alignment of the amino acid sequences of the motifs conserved in the C-terminal regions of PpDof3, PpDof4, CDF1–CDF3 and CDF5. Amino acid residues that are completely conserved and highly conserved are indicated in dark and light grey, respectively. The position of K253, a critical amino acid residue in CDF1 required for the interaction with FKF1, is indicated by an arrow.

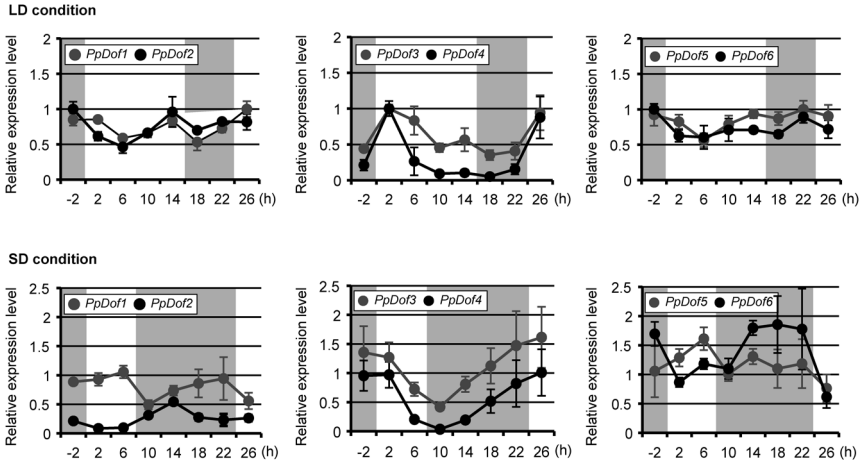

Figure 2. Expression of *PpDof1–PpDof6* genes under long (LD) and short day (SD) conditions. RNA was prepared from protonemal colonies sampled every 4 h for 28 h. Grey shading indicates the night period. Time (h) is expressed as hours after dawn. Values are the mean±SD of three to six biological replicates relative to the levels of 18S rRNA. The maximum expression level of each gene under LD conditions was assigned a reference value of 1 unit.

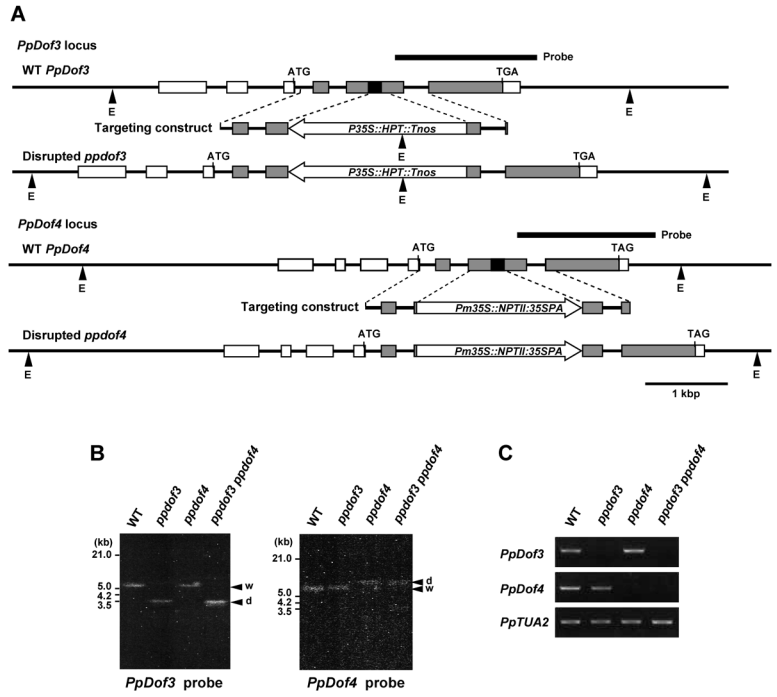

Figure 3. Targeted disruption of *PpDof3* and *PpDof4* genes. (A) Schematic representation of the genomic organization of the wild type and disrupted *PpDof3* and *PpDof4* loci and the gene-targeting vectors. Exons and introns are indicated by boxes and bars, respectively. 5′ and 3′ untranslated regions, coding regions and the sequence encoding the Dof domain are indicated by white, hatched and black boxes, respectively. Positions of DNA probes used in genomic Southern blot analysis are indicted by red bars. E, *Eco*RV site; P35S::*HPT*::Tnos, hygromycin-resistance cassette; Pm35S::*NPTII*:: Tnos, neomycin-resistance cassette. (B) Genomic Southern blot analysis using *Eco*RV-digested genomic DNA from the wild-type (WT), single disruptant (*ppdof3* and *ppdof4*) and double disruptant (*ppdof3 ppdof4*) lines. PpDof3- and PpDof4-specific DNA probes were used. The sizes of DNA fragments originating from the wild type and disrupted *PpDof3* were approximately 6.6 kb and 3.4 kb, respectively, whereas the sizes of DNA fragments originating from the wild type and disrupted *PpDof4* were approximately 7.2 kb and 8.7 kb, respectively. Positions of DNA fragments from the wild type (w) and disrupted (d) *PpDof* gene loci are indicated by arrowheads. (C) RT-PCR analysis of *PpDof3* and *PpDof4* transcripts in the wild-type (WT) and disruptant lines. Transcripts of *PpTUA2* (accession number AB096719) were used as a control.

and SDs. This expression pattern is similar to those of *CDF* genes of flowering plants, including Arabidopsis *CDFs* (Fornara et al. 2009; Imaizumi et al. 2005), the rice homologue *Rdd1* (Iwamoto et al. 2009) and the potato homologues *StCDF1.1* and *StCDF1.2* (Kloosterman et al. 2013). Although CDF4 and COG1 were also reported to display diurnal expression patterns, their patterns are different from those of *CDF1-3* and *CDF5* (Fornara et al. 2009). Hence, *PpDof3* and *PpDof4* present characteristics similar to *CDF*s in terms of mRNA expression pattern as well as protein structure.

Targeted disruptions of PpDof3 *and* PpDof4 *did not affect the expression of* PpCOLs

To investigate the effects of disruption of *PpDof3* and *PpDof4* on *PpCOL* expression, we generated *ppdof3* and *ppdof4* disruptants by gene targeting (Figure 3). We generated single disruptant lines and *ppdof3 ppdof4* double disruptant lines using two selectable marker genes (Figure 3A) due to their possibly redundant roles. Southern blot analysis with genomic DNA from these disruptant lines revealed the presence of single bands of the expected sizes (Figure 3B). Furthermore, RT-PCR analysis revealed that each gene disruption caused the corresponding intact transcript to be undetectable (Figure 3C). Although we could not find any apparent phenotype of these disruptants, we examined the expression of three *PpCOL* genes in these disruptant lines. As reported previously (Shimizu et al. 2004; Zobell et al. 2005), *PpCOL1* transcript levels increased at dawn and remained high throughout the day but were much lower in the dark in the wild type (WT) under both LDs and SDs (Figure 4). This expression pattern of *PpCOL1* was undistinguishable from those of *PpCOL2* and *PpCOL3*. It is worth noting that the expression patterns of the *PpCOL*s were also similar to that of *CrCO* (Serrano et al. 2009) but different from that of *CO*. In Arabidopsis, *CO* transcripts primarily accumulate during the dark period under SDs, whereas *CO* is expressed at moderate to high levels at dawn and dusk under LDs (Yanovsky and Kay 2002). Furthermore, similar to *CrCO* (Serrano et al. 2009), the absolute levels of *PpCOL* transcripts were higher under SDs (Figure 4). Importantly, we found that disruption of *PpDof3* and *PpDof4* did not exert any significant effect on the expression patterns and levels of any *COLs* under SDs or LDs, indicating that PpDof3 and PpDof4 are not involved in controlling the expression of *COLs* in *P. patens*.

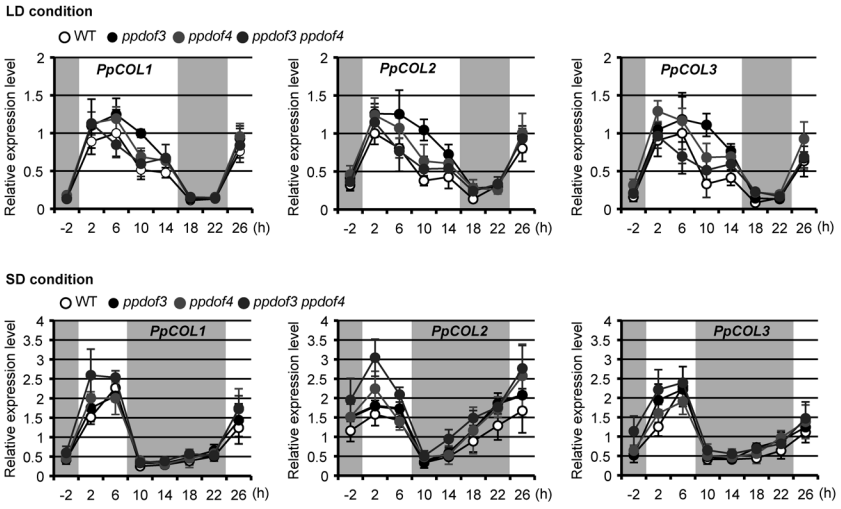

Figure 4. Expression of *PpCOL1–PpCOL3* in wild-type (WT) and *ppdof* mutant *P. patens* under long day (LD) and short day (SD) conditions. RNA was prepared from protonemal colonies sampled every 4 h for 28 h. Grey shading indicates the night period. Time (h) is expressed as hours from dawn. Values are the mean±SD of three to six biological replicates relative to the levels of 18S rRNA. The maximum expression level of each gene under LD conditions was assigned a reference value of 1 unit.

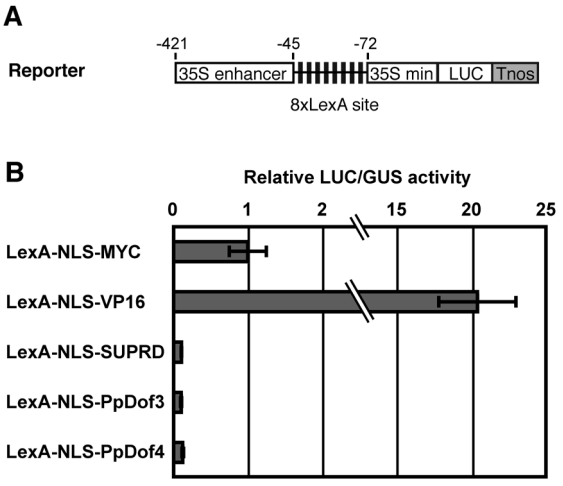

Figure 5. Transcriptional repressor activity. (A) The reporter construct used for the transrepression assay. The reporter construct harbored the *LUC* gene under the control of eight copies of the LexA-binding site (8xLexA site) located between the 35S enhancer and the 35S minimal promoter (35S min). (B) Transrepression assay. The reporter plasmid was co-transfected into maize protoplasts together with an effector plasmid to enable expression of a fusion protein containing the LexA DNA-binding domain, a nuclear localization signal (NLS) and a MYC epitope tag (LexA-NLS-MYC), a VP16 transcriptional activation domain (LexA-NLS-VP16), a repressor domain for SUPERMAN (LexA-NLS-SUPRD), PpDof3 (LexA-NLS-PpDof3) or PpDof4 (LexA-NLS-PpDof4). An internal control plasmid containing the GUS gene under the control of the maize ubiquitin promoter was also co-transfected into the protoplasts. GUS activity values derived from the internal control plasmid were used to normalize LUC activity. Relative LUC activity is shown as the means±SD (n=3). The relative LUC activity obtained with the effector plasmid for the expression of LexA-MYC protein was set at 1.

PpDof3 and PpDof4 are transcriptional repressors

As CDF1 is a transcriptional repressor that directly suppresses *CO* expression (Imaizumi et al. 2005), we examined whether PpDof3 and PpDof4 also function as transcriptional repressors using protoplast transient assays. When we co-transfected expression vectors for PpDof3 and PpDof4 and reporter plasmids containing the *LUC* reporter gene under the control of *PpCOL* promoters, we did not detect any apparent effect of PpDof3 or PpDof4 on LUC activity (Supplemental Figure S1), which is consistent with the fact that disruption of *PpDof3* and *PpDof4* did not affect the expression of *PpCOL*s (Figure 4). Therefore, we next examined the transcription factor activity of PpDof3 and PpDof4 by fusing PpDof3 and PpDof4 to the bacterial DNA-binding protein LexA. We previously succeeded in detecting transcriptional repressor activity of PpDof1 and PpDof2 using this LexA fusion system (Sugiyama et al. 2012). As observed previously (Sugiyama et al. 2012), LexA fused to the VP16 transcriptional activation domain (LexA-VP16) transactivated the LexA-binding site-containing promoter (Figure 5A), while LexA fused to a transcriptional repression domain of the plant transcriptional repressor SUPERMAN (LexA-SUPRD) repressed this promoter. In this assay system, PpDof3 and PpDof4 fused to LexA repressed the expression of *LUC* under the control of a LexA-binding site-containing promoter (Figure 5). The levels of reduced gene expression induced by LexA-PpDof3 and LexA-PpDof4 were comparable to that induced by LexA-SUPRD. Hence, PpDof3 and PpDof4 are transcriptional repressor like Arabidopsis CDF1 protein.

Discussion

Our results indicate that like *CDFs* in flowering plants, the expression of *PpDof3* and *PpDof4* is regulated diurnally (Figure 2), and PpDof3 and PpDof4 function as transcriptional repressors (Figure 5). However, targeted

disruptions of *PpDof3* and *PpDof4* did not affect the expression of *PpCOL*s (Figure 4). Hence, although the diurnal expression of *PpDof3* and *PpDof4*, as well as that of *PpCOL*s, implies that all of these genes are involved in the regulation of light signaling- and/or circadian clock-associated processes, it is likely that CDF-like and CO-like proteins function independently in *P. patens*.

CO and *COLs* are classified into two groups (group I and group II); group I includes *CO*, *CrCO* and all *PpCOLs* as well as *AtCOL1-AtCOL5* (Lagercrantz and Axelsson 2000; Valverde 2011). Since overexpression of *AtCOL1* and *AtCOL2* has little effect on flowering time in Arabidopsis, it was proposed that the roles of CO and COLs do not overlap (Ledger et al. 2001). However, overexpression of *AtCOL5* was recently found to affect flowering time (Hassidim et al. 2009). Furthermore, the *co* mutation in Arabidopsis was complemented by *CrCO* and *BvCOL1*, a sugar beet *COL* that is much more similar to *AtCOL2* than to *CO* (Chia et al. 2008; Serrano et al. 2009). Thus, *CO* and *COLs* belonging to group I were likely derived from an original gene in the common ancestor of algae, bryophytes and flowering plants, and they appear to be closely related, although CO might have evolved to play a unique role in the floral transition in flowering plants. Since the expression of *COLs* is independent of the function of CDF-like Dof proteins in *P. patens*, and since the diurnal expression patterns of the *PpCOL*s were similar to that of *CrCO* but different from that of *CO* in Arabidopsis, it is unlikely that CDF-like Dof proteins had played a role in controlling the expression of the original gene of group I *CO*/*COL* genes in the common ancestor. CDFs might have been recruited and integrated into the CO-mediated regulatory pathway during evolutionary processes to generate the mechanism underlying the accurate regulation of *CO* expression. However, at this stage, we could not exclude the possibility that the regulatory role of CDF-like proteins in controlling expression of *COL*s was lost in *P. patens*.

Interestingly, the expression levels of the rice *CO* homologue *Heading date 1* (*Hd1*) is not modified in transgenic rice plants in which a transgene for a rice homologue of CDF, *rice Dof daily fluctuations 1* (*Rdd1*), was expressed in sense or antisense orientation (Iwamoto et al. 2009). Thus, it is currently unclear that the CDF-meditated regulation of *CO* expression occurs in all flowering plant species. Furthermore, it was very recently reported that *CDF* homologues from tomato function as transcriptional regulators involved in responses to drought and salt stress as well as flowering-time control in Arabidopsis (Corrales et al. 2014). This unexpected function of tomato CDFs, which is probably independent of the modulation of *CO* levels, suggests that CDFs may play another role in flowering plants in addition to the control of flowering. CDF-like Dof proteins may similarly play this additional role in *P. patens*. Identification of the physiological roles of CDF-like Dof proteins in *P. patens* would be necessary to evaluate this speculation. It is noteworthy that in moss, reproductive development begins with the generation of gametophores (Cove 2005), which is promoted by short day lengths (Hohe et al. 2002). However, *P. patens* lacks an FT homologue (Holm et al. 2010), and the role of PpCOL in reproductive development have not been shown yet.

In flowering plants, CDFs interact with FKF1 and GI (Imaizumi et al. 2005; Kloosterman et al. 2013; Sawa et al. 2007). Thus, we also examined whether PpDof3 and PpDof4 have the potential to interact with Arabidopsis FKF1 and GI using yeast two-hybrid assays (Supplemental Figure S2). The results indicate that unlike CDF1 from Arabidopsis, PpDof3 and PpDof4 did not interact with FKF1 or GI (Supplemental Figure S2). The mutation of a lysine residue within the conserved amino acid motif that characterizes CDFs (the 253th amino acid residue of CDF1) markedly reduces the interaction with FKF1, suggesting that this lysine residue plays a critical role in the interaction with FKF1 (Imaizumi et al. 2005). This lysine residue is not conserved in PpDof3 or PpDof4 (Figure 1b), which is consistent with the lack of an apparent interaction between PpDof3 and PpDof4 and FKF1. The GI-FKF1 system to regulate growth-phase transition was very recently found in the liverwort *Marchantia polymorpha* (Kubota et al. 2014). As *P. patens* lacks homologues of FKF1 and GI (Holm et al. 2010), identification and characterization of CDF-like Dof proteins and COLs in *M. polymorpha* might provide a new clue to understand the origin of the CO-mediated regulatory pathway.

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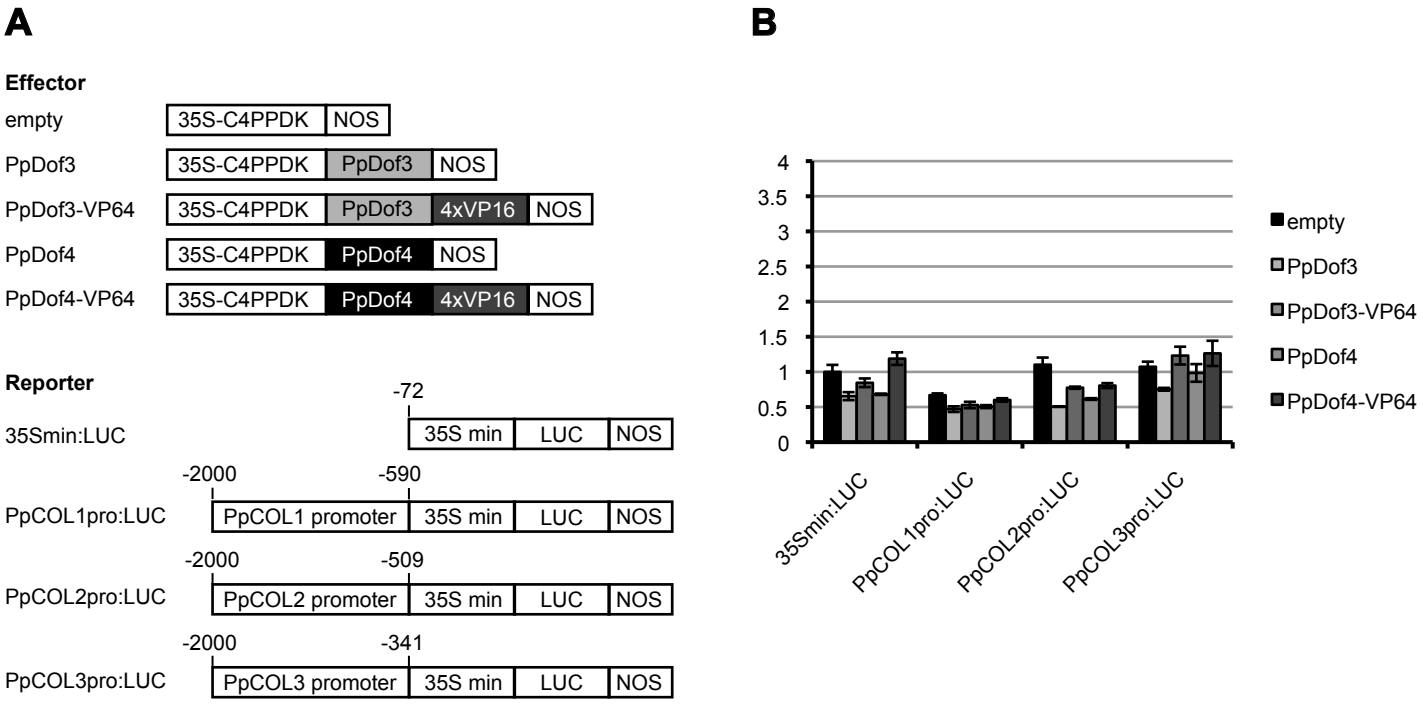
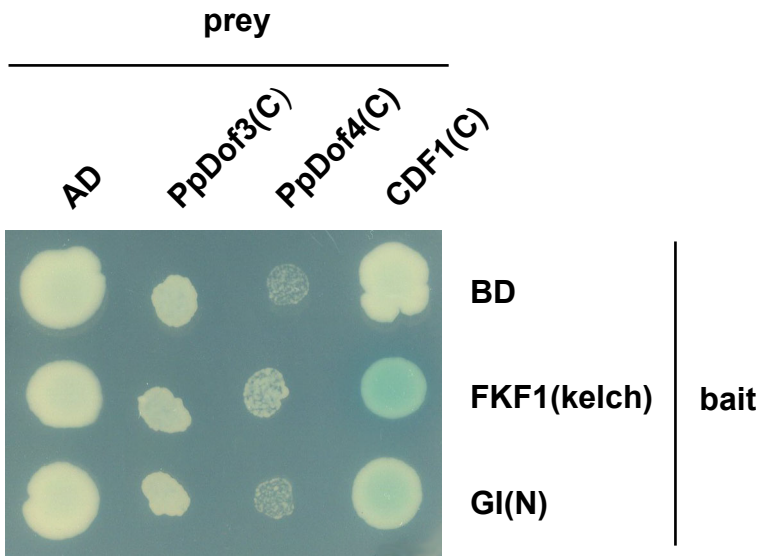


Supplemental Figure S1. Co-transfection experiment using PpDof3 and PpDof4 expression vectors and reporter constructs containing LUC under the control of PpCOL promoters. (A) Schematic representation of effector and reporter constructs. In the effector plasmids, coding regions for PpDof3 and PpDof4 cDNA (obtained by PCR using gene-specific primers; Supplemental Table S1) were inserted between the 35S-C4PPDK promoter and the terminator of the nopaline synthase gene or the 35S-C4PPDK promoter and the sequence for four copies of the VP16 transcriptional activation domain (VP64). In the reporter plasmids, the DNA fragments for the PpCOL1 promoter (−2000 to −590, relative to the translational start codon), the PpCOL2 promoter (−2000 to −509) or the PpCOL3 promoter (−2000 to −341) obtained by PCR using gene-specific primers (Supplemental Table S1) were placed upstream of the 35S minimal promoter, which was truncated at position −72 (35S min). 35S-C4PPDK, a strong constitutive promoter generated by fusing the 35S enhancer to the minimal promoter sequence from the maize C4PPDK gene promoter (Sheen 1990 Plant Cell 2: 1027-1038); PpDof3, the entire coding sequence for PpDof3; PpDof4, the entire coding sequence for PpDof4; VP16, sequence for the VP16 transcriptional activation domain; NOS, terminator of the nopaline synthase gene; LUC, luciferase gene. (B) Protoplast transient assays. The reporter plasmid and an effector plasmid were co-transfected into protoplasts together with an internal control plasmid containing the GUS gene under the control of the maize ubiquitin promoter. LUC activity was normalized using GUS activity, and relative LUC activity is shown as the means ± SD (n = 3). The relative LUC activity, which was obtained with the combination of 35Smin:LUC reporter and empty effector plasmids, was set at 1.



Supplemental Figure S2. Yeast two-hybrid assay. Yeast transformants were plated on SD-L-W plates containing X-α-Gal (Clontech, Mountain View, CA, USA). An interaction between bait and prey proteins induces the expression of the gene for α-galactosidase, which hydrolyses X-α-Gal, thereby causing the colonies to turn blue. AD, GAL4 activation domain; PpDof3(C), C-terminal region of PpDof3 (209–593 aa); PpDof4(C), C-terminal region of PpDof4 (210–593 aa); CDF1(C), C-terminal region of CDF1 (108–298 aa); BD, GAL4 DNA-binding domain; FKF1 (kelch), kelch repeats domain of FKF1; GI(N), N-terminal region of GI. We used the same regions that were used as the kelch repeat domain of FKF1 and the N-terminal region of GI in previous reports by Imaizumi et al. (2005 Science 309: 293-297) and Sawa et al. (2007 Science, 318: 261-265). For unknown reasons, the expression of the C-terminal regions of PpDof3 and PpDof4 caused growth inhibition in yeast.

Table S1. List of PCR primers

Target	Forward (5' > 3')	Reverse (5' > 3')
Gene disruption & Southern blot analysis		
PpDof3 genomic clone	GAAATGAACCTTGCGTTTCAGTG	CCAGAATCACCAGAGACGTTCAC
PpDof4 genomic clone	TTGATCCGACTTTCACTTTCTGC	ACATTCGTTCGAGAAGGAGAACC
PpDof3-specific probe	ATCACTAGCCTCCCCAGTAGTCAAG	ACCTGAAAGCGAGATTCCATAAAAG
PpDof4-specific probe	AGCAGTCCCAGTAATCTCAATAGCC	GAAACTTGGAGTGGACAACTGAAGA
Promoter regions for reporter constructs		
PpCOL1 promoter	AAGGATCCTACCATTGGTGAGATCCAGT	TTCTCGAGAGGCTTTTGTTTTCCGGGCA
PpCOL2 promoter	TTGGATCCAGTGAACCCAGGCTCGCT	TTCTCGAGCGAGACGATCCAGGCT
PpCOL3 promoter	TAGGATCCGGTTCGAGTGTAAATTCTGT	TTCTCGAGAATGGAGCGAGGTGAGGAA
Coding regions of PpDof3 and PpDof4 for effector plasmids		
LexA-NLS-PpDof3	TACCCGGGATGATGATATCCAATGTCAAAC	TTCTGCAGTCATGATTTCACTTGCTTCC
LexA-NLS-PpDof4	TACCCGGGATGAGGATGATGTCCCATGT	TTCTGCAGCTATGATGTGTCAGATGCTT
PpDof3	TACGGATCCATGATGATATCCAATGTCAAAC	TTCTGCAGTCATGATTTCACTTGCTTCC
PpDof4	TACGGATCCATGAGGATGATGTCCCATGTCAAAC	TTCTGCAGCTATGATGTGTCAGATGCTT
PpDof3-VP64	TACGGATCCATGATGATATCCAATGTCAAAC	TACCCCGGGTGATTTCACTTGCTTCCTATCA
PpDof4-VP64	TACGGATCCATGAGGATGATGTCCCATGTCAAAC	TACCCCGGGTGATGTGTCAGATGCTTTGCT
qPCR		
PpDof1	TCGCCTCACCCAACCAAACCG	ATGCGCGCGTAGAGAACTCCG
PpDof2	ATCGCCTTGTTAGCGTCCAT	ACAATAACCTCGCCTCGCAA
PpDof3	TGGACCTACTCATGCCAACACAGT	TCGGTTGTTGTCCCAGAATCACCT
PpDof4	TGGACCTGCTCATGTCAACATGGC	TCGGCTGTAGCTCCTCTCGCCTTT
PpDof5	CTGCAGGGGGATGGACTGGGA	TGCAGCACTCCAACCACTCGT
PpDof6	GTAGTGCTTTGAGCGGGGGCT	AGCAGGCACTGCATTCGCAA
PpCOL1	ACATGGAGCAGTTTGGCTCA	GCTGCCACTTTTTCAGGTGC
PpCOL2	TCGGAGACGCTAAGTCATGC	GGAGGAATGTGCTGCTCCAT
PpCOL3	GACCATCCGGTACGCTTCAA	GGAACCACCCCAAAACCTGA
18S rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Semi-quantitative PCR		
PpDof3	TGGTCACACCTTTTCCTCTTTCTTC	GTACTCGGGCTGCTGGAACTT
PpDof4	AGAGATGTTGAAGGGCGAGTAGC	GATTACTGGGACTGCTGGCATTA
PpTUA2	TGTGCTGCTGGATAATGAAGCG	CTCGTGCTGTTCGAAATCATGC