Photoperiod-dependent regulation of cell growth by *PpCCA1a* and *PpCCA1b* genes encoding single-myb clock proteins in the moss *Physcomitrella patens*

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The *PpCCA1a* and *PpCCA1b* genes of the moss *Physcomitrella patens* are functional homologs of the *Arabidopsis thaliana* circadian clock genes *CCA1/LHY*. We made use of disruptant lines for *PpCCA1a* and/or *PpCCA1b* to elucidate the physiological significance of these genes in the growth of moss protonemal tissue under alternating day/night cycles. Protonemal cells of the double disruptant line, carrying neither of the two genes, grew faster than those of the wild-type plant (WT) in long days (LD), whereas no difference in the growth rate was detected between them in short days (SD). The double disruptant line also showed day length-dependent phenotypic changes in the *PpCCA1b* promoter activity: the diurnal profile of bioluminescence from the *P_{CCA1b}::LUC+* reporter strain was more significantly affected in LD than in SD. These observations are the first demonstration of a physiological function of the circadian clock in non-angiosperm land plants, and are consistent with recent findings that the clock controls hypocotyl elongation of *A. thaliana* in a photoperiod-dependent manner.

Key words: circadian clock, circadian rhythm, clock genes, photoperiod, *Physcomitrella patens*

INTRODUCTION

Circadian rhythms are endogenous biological oscillations with a ~24-h period length, and they are controlled by an autonomous oscillator, the circadian clock (Edmunds, 1988). The primary role of this clock is to adjust the timing of metabolism, physiology and behavior of organisms, coordinating them to environmental factors that cycle with a ~24-h period length (Edmunds, 1988; Pittendrigh, 1993). Recently, molecular genetic studies have led to the isolation of many genes with critical functions in regulation of the clock oscillation in various model organisms (Hamilton and Kay, 2008). In the dicot model plant *Arabidopsis thaliana*, best characterized examples are *Circadian Clock-Associated 1* (*CCA1*) and its paralog *Late elongated HYpocotyl* (*LHY*), which encode single-myb transcription factors (Wang and Tobin, 1998; Schaffer et al., 1998; Carré and Kim, 2002). *CCA1* and *LHY* abolish the clock oscillation when overexpressed singly, and shorten the period length of the clock when nullified singly or in pair (Wang and Tobin, 1998; Schaffer et al., 1998; Green and Tobin, 1999; Mizoguchi et al.,

2002; Alabadí et al., 2002; Ding et al., 2007), and they are considered to be “clock genes”, critical components of the core clock machinery. The *A. thaliana* clock mechanism is proposed, largely based on genetic data from many mutants, to be founded on interlocked regulatory loops between *CCA1/LHY* and several other types of clock genes (Mizuno and Nakamichi, 2005; Gardner et al., 2006; McClung, 2008; Harmer, 2009).

In a previous study, we characterized two genes *PpCCA1a* and *PpCCA1b*, homologs of *CCA1/LHY* in the moss *Physcomitrella patens* (Okada et al., 2009). The accumulation of *PpCCA1a* and *PpCCA1b* mRNAs showed diurnal rhythms in 24-h light-dark cycles, and relative positions of their peaks and troughs in the light-dark cycles are similar to those of *CCA1/LHY* of *A. thaliana*. The double disruptants for *PpCCA1a* and *PpCCA1b* showed: 1) short periodicity and damped amplitude in the *PpCCA1b* rhythm; 2) similar changes in other rhythmically expressed genes *PpSIG5* and *PpPRRa*; 3) de-repression of *PpCCA1b* transcription levels, indicating a negative feedback regulation. These observations indicate that the two genes are not merely structural homologs but also functional counterparts of *CCA1/LHY* (Okada et al., 2009). It has been a mystery, however, whether these clock genes as well as the clock itself have

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any physiological functions in *P. patens* cells.

In the current study, we examine the physiological significance of the two moss clock genes under day-night alterations. We compared: 1) growths of protonemal colonies, and 2) the *PpCCA1b* promoter activity as a marker for transcriptional regulation by the clock, between the wild-type plant (WT) and single or double disruptants for *PpCCA1a/PpCCA1b*. Our results indicate that *PpCCA1a/PpCCA1b* have photoperiod-dependent regulatory functions in the growth of moss protonemal tissues.

MATERIALS AND METHODS

Plant materials and growth conditions *Physcomitrella patens* ssp. *patens* (Ashton and Cove, 1977) was maintained in 12-h:12-h light-dark cycles using white fluorescent lamps as the light source (light intensity: $\sim 40 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C. Protonemal tissues, which were grown on BCDAT medium supplemented with 1 mM CaCl_2 (Nishiyama et al., 2000), were collected every five days, and were ground with a homogenizer before they were applied to new BCDAT agar plates.

Monitoring of bioluminescence and data analysis

Bioluminescence from the reporter strains were monitored as previously described using a photomultiplier tube-based apparatus (Aoki et al., 2004; Okamoto et al., 2007). The bioluminescence rhythms from the *P_{CCA1b}::LUC+* reporter strain in DD are similar in their persistence and amplitude at glucose concentrations from 0 to 0.5%, whereas their amplitude became lower when higher glucose concentrations were used. Therefore, a glucose concentration of 0.5% was used for bioluminescence monitoring. Rhythm data were analyzed with the Biological Rhythm Analysis Software System (BRASS; Southern et al., 2006), available at <http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm>.

Observations of the growth of colonies As starting materials for the observation of colony sizes, protonemal colonies (~ 2 mm in diameter) were inoculated the same distance apart on the agar plates. The diameters of the colonies were measured, and they were observed with a stereomicroscope, and fine observations were made using a microscope (BX50W1, Olympus, Tokyo).

RESULTS AND DISCUSSION

We observed the growths of protonemal colonies of the wild-type strain (WT) and *PpCCA1a/PpCCA1b* disruptants under long days (LD; 16-h:8-h light-dark cycles) or short days (SD; 8-h:16-h light-dark cycles) (Fig. 1). In this study, WT and the *PpCCA1a/b* disruptants indicate the wild-type reporter strain *P_{CCA1b}::luc+* and its deriva-

tives (in which *PpCCA1a* and/or *PpCCA1b* are disrupted), respectively (Okada et al., 2009). We compared sizes (diameters) of protonemal colonies after inoculation of cells onto agar plates at intervals shown in Fig. 1a. In LD, the double disruptant showed colony sizes different from those of WT. In the first week, no difference was observed between WT and the double disruptant. On the 17th day, colonies of the double disruptant were 1.4-fold larger than those of WT, and this diameter ratio slightly increased to 1.5-fold in the next 10 days. The sizes and shapes as well as branching patterns of cells were similar between WT and the double disruptant in LD (Fig. 2), indicating that it was only the growth rate which was affected in the disruptant. Therefore, in LD, the growth rate of the double disruptant relative to that of WT largely increased in the period from the 7th to the 17th day. Two single disruptants showed growths with no significant difference to WT over the observation period in LD. In SD, on the other hand, the size, shape and color of colonies of any of the disruptants were indistinguishable from those of WT as well as from one another (Fig. 1a, right; Fig. 1c). Together, these observations indicate that *PpCCA1a/PpCCA1b* control the growth rate of protonemal cells in a day length-dependent manner in *P. patens*.

We also compared the bioluminescence profiles of WT and disruptants in LD or in SD. In WT, bioluminescence rhythms similar to those observed in 12-h:12-h light-dark cycles were observed (Okada et al., 2009; Fig. 3, open circles). It should be noted that the peaks of the *PpCCA1b* rhythm occurred before dawn in SD, because the peaks of *A. thaliana CCA1/LHY* occur, to our knowledge, always at or immediately after dawn in light-dark cycles. The *LpLHYH1* gene, a duckweed (*Lemna paucicostata*) homolog of *CCA1/LHY*, also showed a peak before dawn in SD but not in LD (Miwa et al., 2006). In the double disruptant, bioluminescence profiles were affected both in LD and SD, their extents being greater in LD than in SD. First, the amplitude of the diurnal bioluminescence profiles was lower in the double disruptant than in WT. In LD, the double disruptant showed a 30% lower amplitude than that of WT: the mean peak-to-trough ratios for WT and the double disruptant are 1.66 ± 0.06 ($n = 8$) and 1.17 ± 0.06 ($n = 7$), respectively. In SD, on the other hand, the double disruptant showed a 19% lower amplitude than that of WT: the mean peak-to-trough ratios for WT and the double disruptant are 1.86 ± 0.08 ($n = 14$) and 1.50 ± 0.08 ($n = 11$), respectively. Next, the trough positions in the double disruptant were advanced relative to those in WT. In LD, troughs of the double disruptant were observed 3.3-h earlier than those of WT: they occurred at $\text{ZT}10.5 \pm 0.7$ ($n = 8$) and $\text{ZT}7.2 \pm 0.8$ ($n = 7$) in WT and the double disruptant, respectively (ZT is for Zeitgeber Time, which represents time in a light dark cycle, putting the timing for the light onset as

Fig. 1. Growth of protonemal colonies in *PpCCA1a/PpCCA1b* disruptants under LD and SD. (a) The sizes of protonemal colonies are compared between WT, the *PpCCA1a* single disruptant ($\Delta cca1a$), the *PpCCA1b* single disruptant ($\Delta cca1b$) and the *PpCCA1a PpCCA1b* double disruptant ($\Delta cca1a cca1b$). The diameters of the colonies were measured after 7 days (open bars), 17 days (light grey bars) and 27 days (dark grey bars) after protonemal cells of each strain were inoculated into a small colony with diameter of ~2 mm on agar plates. The colonies were grown in LD (left-hand graph) or in SD (right-hand graph). Each value is shown with means $\pm$ SD from at least seven colonies, and all the experiments were repeated at least twice with similar results. Also shown are photos of the representative colony of each strain grown in LD (b) or in SD (c).

Fig. 2. Microscopic observations of protonemal colonies of WT and the double disruptant. Protonemal colonies of WT (left; A and C) and the double disruptant (right; B and D) were microscopically observed after the colonies grew for 27 days in LD. Photos taken at the same magnitude are aligned next to each other.

ZT0). In SD, on the other hand, troughs of the double disruptant were observed 1.8-h earlier than those of WT: they occurred at $ZT6.7 \pm 0.9$ ($n = 14$) and $ZT4.9 \pm 1.1$ ($n = 11$) in WT and the double disruptant, respectively. In LD, the bioluminescence profile of the *PpCCA1b* single disruptant showed changes similar to those of the double disruptant albeit to a less extent (Fig. 3a, middle), whereas the *PpCCA1a* single disruptant showed only a trace of amplitude lowering (Fig. 3a, top). In SD, the two single disruptants showed little difference, if any, in their bioluminescent profiles to those simultaneously measured from WT (Fig. 3b, top and middle).

Dowson-Day and Millar (1999) reported that the circadian clock controls hypocotyl elongation with alternating daily phases of rapid growth and growth arrest in *A. thaliana*. The daily growth arrest is abolished in a conditionally arrhythmic mutant *early flowering 3-1* (*elf3-1*), resulting in the long-hypocotyl phenotype of this mutant (Dowson-Day and Millar, 1999). Their observations, enhanced growth rates resulting from dysfunction of the clock, are consistent with the larger colony sizes of the disruptant of the moss clock genes observed in this

Fig. 3. Temporal profiles of the *PCCA1b* promoter activity in *PpCCA1a/PpCCA1b* disruptants under LD and SD. Protonemal cells of each strain were entrained for more than two weeks and then their bioluminescence was measured in LD (a) or in SD (b). Closed triangles (top), closed squares (middle) and closed circles (bottom) represent bioluminescence from the *PpCCA1a* single disruptant, the *PpCCA1b* single disruptant and the *PpCCA1a PpCCA1b* double disruptant, respectively. Open circles represent bioluminescence from WT, measured in parallel with each disruptant. Data were detrended and normalized by BRASS (Southern et al., 2006) as in a previous study (Okada et al., 2009), and the averages and standard deviations of at least four independent samples are plotted. Open and closed bars denote light and dark periods, respectively, in each graph.

study. While Dowson-Day and Millar (1999) observed a correlation between long hypocotyls and the absence of rhythmic growth arrest in continuous light (LL), we observed significant colony enlargement of the *PpCCA1a PpCCA1b* double disruptant in light-dark cycle conditions. Although the *PpCCA1a PpCCA1b* double disruptant showed colony sizes larger than those of WT also in LL, the enlargement is less conspicuous in LL than in LD (Table 1). Nozue et al. (2007) studied the molecular mechanism of diurnal rhythms of hypocotyl elongation in light-dark cycle conditions, and they demonstrated that normal daily growth patterns depend on interactions between internal (circadian) and external (light) cues. According to their model, hypocotyl elongation is “gated” to occur before dawn, when the internal and external cues coincide with each other. More recently, Niwa et al. (2009) demonstrated that hypocotyl elongation is not only clock-controlled but also under photoperiod-dependent control. They showed that the “gate” of hypocotyl elon-

gation is open only in SD but not in LD, depending on whether a coincidence occurs or not between internal and external cues. In our double disruptant, which is not arrhythmic and shows only partial phenotypic changes in properties of the clock (Okada et al., 2009), such a “gate” might have been lengthened through disturbance of the clock by the disruption of *PpCCA1a/PpCCA1b*, causing abnormal synchronization to external light-dark cycles in LD. This idea is consistent with the result that the profile of transcriptional activity of *PpCCA1b* is more seriously affected in LD than in SD (Fig. 3a, bottom).

The advancement of trough positions in the *PpCCA1b* transcriptional activity in the double disruptant is consistent with observations by Mizoguchi et al. (2002). They reported that profiles of diurnal expression rhythms of clock-controlled genes were affected by inactivation of both *CCA1* and *LHY* in *A. thaliana*: the troughs in morning-expressed genes *COL1* and *LHYL1*, as well as the peaks in evening-expressed genes *GI* and *TOC1*, were

Table 1. Growth of protonemal colonies under various light regimen

Photoperiod	Diameter of protonemal colony (mm)			
	WT	<i>Acca1a</i>	<i>Acca1b</i>	<i>Acca1a cca1b</i>
VSD	4.8 ± 0.5 (13)	4.8 ± 0.5 (12)	5.2 ± 0.5 (14)	5.2 ± 0.9 (15)
SD	8.1 ± 0.7 (9)	7.5 ± 0.6 (9)	7.6 ± 0.6 (9)	7.6 ± 0.5 (10)
LD	11.0 ± 1.3 (11)	11.3 ± 0.8 (10)	11.8 ± 1.4 (9)	16.3 ± 1.2 (9)**
HLD	15.4 ± 1.1 (7)	15.6 ± 0.9 (7)	15.6 ± 0.8 (9)	18.9 ± 1.4 (12)**
LL	18.3 ± 0.6 (9)	19.3 ± 1.0 (8)	18.6 ± 1.0 (9)	20.1 ± 0.6 (9)*

The sizes of protonemal colonies grown under various light conditions are summarized. VSD, very short days (4-h:20-h light-dark cycles); SD, short days (8-h:16-h light-dark cycles); LD, long days (16-h:8-h light-dark cycles); HLD, high-intensity light long days (7-h:9-h:8-h normal light/high-intensity light-dark cycles); LL (continuous light). In HLD, the latter 9 h of the light period of LD, roughly corresponding to the period when bioluminescence profiles diverged between WT and the double disruptant, was illuminated with high-intensity light at a fluence rate of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The diameters of the colonies were measured after growth for 27 days (20 days only for VSD) on agar plates. Each value is shown with means $\pm$ SD (the numbers of colonies examined are in parentheses), and all the experiments were repeated at least twice with similar results. The significance of differences between the control (WT) and the disruptants were determined with the two-tailed multiple t-test with Bonferroni correction following ANOVA (3 comparisons in 4 groups). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, vs. control.

advanced in the *CCA1 LHY* double mutant (Mizoguchi et al., 2002). Because *PpCCA1a* and *PpCCA1b* feed back to repress their own expression (Okada et al., 2009) as reported for *CCA1* and *LHY* (Schaffer et al., 1998; Wang and Tobin, 1998), reactivation of the promoter activity of *PpCCA1b* would start earlier in the double mutant than in WT, and this might have caused the advancement of trough positions.

The circadian clock can affect gross morphological phenotypes of a plant in a different way. Dodd et al. (2005) reported that plants with periods that match environmental cycles showed, compared to those with periods that do not, whether mutants or WT, faster growth along with several other advantageous phenotypic changes. Matching the endogenous clock period with the period of exogenous light-dark cycles, named “circadian resonance”, probably provides an advantage by optimizing the phase relation between various clock-controlled parameters and exogenous day-night cycles (Ouyang et al., 1998). The WT *P. patens* cells have a clock with a ~22.8 h-period length, which is closer, hence more resonant, to the length of the light-dark cycle (24 h) than that of the double disruptant (21.1 h) (Okada et al., 2009). However, we could not observe an effect of circadian resonance in our experimental conditions (Fig. 1; Table 1). Green et al. (2002) reported that plants in which the *CCA1* gene was overexpressed were less viable under very short-days (4 h-light:20 h-dark cycles) than their WT counterparts. Therefore, we additionally observed the growth of colonies under very short days, however, no significant difference was observed between WT and the double disruptant (Table 1). We also tested the effect of high-intensity light to simulate stressful daytime conditions in nature, but could not detect any difference either (Table 1). Given the relatively small differences in the period

lengths of WT and the moss disruptant, longer-term experiments, possibly over multiple generations, would be needed in order to detect the advantageous effect of circadian resonance in *P. patens*.

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