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Research Letter – Physiology, Biochemistry and Genetics

Multiple calcium signaling genes play a role in the circadian period of *Neurospora crassa*

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Abstract

The Ca^{2+} signaling genes *cpe-1*, *plc-1*, *ncs-1*, *splA*$_2$, *camk-1*, *camk-2*, *camk-3*, *camk-4*, *cmd*, and *cnb-1* are necessary for a normal circadian period length in *Neurospora crassa*. In addition, the Q_{10} values ranged between 0.8 and 1.2 for the single mutants lacking *cpe-1*, *splA*$_2$, *camk-1*, *camk-2*, *camk-3*, *camk-4*, and *cnb-1*, suggesting that the circadian clock exhibits standard temperature compensation. However, the Q_{10} value for the Δ*plc-1* mutant was 1.41 at 25 and 30 °C, 1.53 and 1.40 for the Δ*ncs-1* mutant at 20 and 25 °C, and at 20 and 30 °C, respectively, suggesting a partial loss of temperature compensation in these two mutants. Moreover, expression of *frq*, a regulator of the circadian period, and the blue light receptor *wc-1*, were increased >2-fold in the Δ*plc-1*, Δ*plc-1*; Δ*cpe-1*, and the Δ*plc-1*; Δ*splA*$_2$ mutants at 20 °C. The *frq* mRNA level was increased >2-fold in the Δ*ncs-1* mutant compared to the *ras-1*^{bd} strain at 20 °C. Therefore, multiple Ca^{2+} signaling genes regulate the circadian period, by influencing expression of the *frq* and *wc-1* genes that are critical for maintaining the normal circadian period length in *N. crassa*.

Keywords: calcium signaling, circadian clock, *Neurospora crassa*, period length, *frequency*, *white collar-1*

Introduction

Circadian rhythms are ubiquitous biological oscillations with an ~24 h period that impact diverse cell processes, including cell division, homeostasis, immunity, physiology, and sleep-wake cycles in eukaryotes ranging from fungi to mammals (Aronson et al. 1994a). This internal timekeeping mechanism or daily clock phenomenon was termed "circadian" in the 1950s by Halberg, combining the Latin terms '*circa*' for 'about' and '*dien*' for 'day' to explain the biological activity having a frequency of one cycle in every 24 h (Halberg et al. 2003). The circadian clock has a free-running period, which runs at approximately the same rate within a broad range of temperatures, a phenomenon known as temperature compensation, expressed as Q_{10} that normally ranges between 0.8 and 1.2 (Mattern et al. 1982, Sorek and Levy 2012, Avello et al. 2019).

The calcium ion (Ca^{2+}), a ubiquitous secondary messenger, plays a role in mammalian circadian timekeeping (O'Neill and Reddy 2012). The extracellular signals mediated by Ca^{2+} regulate amplitude, phase, and period in mammals (O'Neill and Reddy 2012). In mice, a transmembrane Ca^{2+} flux maintains the molecular rhythmicity by regulating the expression of the clock gene in the hypothalamic suprachiasmatic nucleus (SCN; Lundkvist et al. 2005). The circadian timing in the SCN neurons is entrained by changes in adenylate cyclase and phospholipase C (PLC) activities (An et al. 2011). In addition, inhibition of the inositol 1,4,5-trisphosphate receptor (IP_3R) or the endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) increases period length, indicating a role for Ca^{2+} in modulating the molecular circadian clock in the liver of rats (Báez-Ruiz and Díaz-Muñoz 2011). In both prokaryotes and eukaryotes, Na^{2+}/Ca^{2+} mediated Ca^{2+} signaling is conserved in temperature-compensated circadian rhythms (Kon et al. 2021).

The model filamentous fungus *Neurospora crassa* displays a visible circadian rhythm in the vegetative developmental program (Bell-pedersen et al. 1992, Aronson et al. 1994a). In *N. crassa*, the circadian period is changed only slightly at different temperatures, thus compensated for the temperature difference, and expressed as the Q_{10} ratio. The stable interaction of FREQUENCY-casein Kinase 1 (FRQ-CK1) is critical for temperature compensation in *N. crassa* (Hu et al. 2021). Several molecular components of the clock, such as *frequency* (*frq*) and the *white collar* genes (*wc-1* and *wc-2*) have already been characterized (Aronson et al. 1994a, 1994b, Crosthwaite et al. 1997). The *frq* transcript oscillates daily and the FRQ protein sets the circadian clock phase (Aronson et al. 1994b). In *N. crassa*, WC-1 and WC-2 are two proteins in the GATA zinc finger family of nuclear transcription factors that bind to the consensus element within the promoter of light-regulated genes (Ballario et al. 1996, Linden and Macino 1997). The WC proteins play an essential role in maintaining the *N. crassa* circadian feedback loop (Crosthwaite et al. 1997). The WC-1 and WC-2 proteins interact to form the white collar complex (WCC) via their conserved Per-Arnt-Sim (PAS) domains (Ballario et al. 1998, Cheng et al. 2002, Franchi et al. 2005, Wang et al. 2016) to maintain circadian rhythmicity in constant darkness by regulating rhythmic expression from the *frq* locus (Crosthwaite et al. 1997, Garceau et al. 1997). The newly synthesized FRQ is progressively phosphorylated by several kinases and regulated by phosphatases (Baker et al. 2012). FRQ inhibits its own transcription through FRQ-dependent phosphorylation of

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the WCC complex, using a negative feedback loop, because the phosphorylated WCC complex cannot bind to the *frq* promoter (Aronson et al. 1994b, Baker et al. 2012, Wang et al. 2019). However, when FRQ levels fall below a critical threshold, *frq* transcription is reactivated (Garceau et al. 1997, Liu and Bell-Pedersen 2006). Therefore, the negative feedback loop causes daily rhythmic accumulation of *frq* mRNA and FRQ protein, and their oscillations are essential for the normal circadian clock in *N. crassa* (Garceau et al. 1997, Liu and Bell-Pedersen 2006). Moreover, FRQ acts positively on WC-1 and WC-2 by upregulating the WC-1 protein levels post-transcriptionally and *wc-2* mRNA levels transcriptionally, thereby forming an interlocked positive feedback loop (Cheng et al. 2001).

The Ca^{2+} signaling gene *cmd* encodes calmodulin (CaM), a Ca^{2+} sensor required for growth, stress tolerance, circadian clock, and sexual development in *N. crassa* (Laxmi and Tamuli 2015, 2017). CaM activates several other Ca^{2+} signaling proteins, including Ca^{2+}/CaM-dependent kinases (Ca^{2+}/CaMKs) and calcineurin. In *N. crassa*, the *camk-1*, *camk-2*, *camk-3*, and *camk-4* genes encode four different Ca^{2+}/CaMKs, including Ca^{2+}/CaMK-1 and Ca^{2+}/CaMK-2, that are both required for full fertility (Tamuli et al. 2011, Kumar and Tamuli 2014). The *cna-1* and *cnb-1* genes encode the calcineurin catalytic subunit A (CNA-1) and the regulatory subunit B (CNB-1), respectively in *N. crassa* (Tamuli et al. 2016, Kumar et al. 2019).

The Ca^{2+} signaling genes *cpe-1*, *plc-1*, and *splA*$_2$ encode a Ca^{2+}/H^+ exchanger (CPE-1), a phospholipase C-1 (PLC-1), and a secretory phospholipase A_2 (sPLA$_2$), respectively (Barman and Tamuli 2015). The *cpe-1*, *plc-1*, and *splA*$_2$ genes are necessary for growth, conidiation, carotenoid accumulation, and maintaining Ca^{2+} homeostasis in *N. crassa* (Barman and Tamuli 2015, Roy et al. 2020). Moreover, *cpe-1* and *splA*$_2$ exhibit epistatic interactions with *plc-1* for normal asexual and sexual development in *N. crassa* (Barman and Tamuli 2017). In the cell, various Ca^{2+} sensors respond to high concentrations of Ca^{2+}. The neuronal calcium sensor-1 (NCS-1) protein (Deka et al. 2011) interacts with a Ca^{2+}-permeable channel (MID-1), which has a role in maintaining Ca^{2+} homeostasis (Lew et al. 2008), and possibly blocks the channel for tolerance to high Ca^{2+} concentrations (Gohain and Tamuli 2019).

Although the core clock mechanism has been identified in *N. crassa*, a possible role for Ca^{2+} signaling genes in regulating the circadian rhythm has remained largely unexplored. In this study, we investigated multiple Ca^{2+} signaling genes for their roles in regulating *N. crassa* circadian period length under different temperature conditions. We found that the Ca^{2+} signaling genes *cpe-1*, *plc-1*, *ncs-1*, *splA*$_2$, *camk-1*, *camk-2*, *camk-3*, *camk-4*, *cmd*, and *cnb-1* play roles in maintaining the normal circadian period length and/or temperature compensation in *N. crassa*. Additionally, expression of the clock regulatory *frq* and *wc-1* genes was altered in the Ca^{2+} signaling mutants that exhibited abnormal period length.

Materials and methods

Strains, media, and growth conditions

Strains were obtained from the Fungal Genetics Stock Center (FGSC; Kansas State University, Manhattan, KS; McCluskey et al. 2010) or generated in this study (Table S1). For vegetative growth, strains were routinely cultured on Vogel's minimal medium N (VM; Vogel 1964) containing 1.5% D-glucose as a carbon source and 2% Bacto agar (Davis and De Serres 1970). VM was supplemented with calcium-D-pantothenate (CMS168-100GM, Himedia Laboratories, Mumbai, India) at a concentration of 0.5 mg/ml for the growth of the pantothenic acid auxotrophic mutants (*pan-2*$^-$). For the *cnb-1*^{RIP} mutants, 50 μM bathocuproinedisulfonic acid (BCS; B1125-500 MG, Sigma–Aldrich, St. Louis, MO, USA) was added to VM in addition to calcium-D-pantothenate. Crosses were performed using the synthetic crossing medium (SCM; Westergaard and Mitchell 1947), containing 1.5% D-glucose and 2% Bacto agar. Ascospores produced from the crosses were germinated by heat shock at 60 °C for 45 min on Petri dishes containing 0.05% fructose, 0.05% glucose, 2% sorbose (FGS), and 2% Bacto agar, and individual progeny were isolated. We crossed the *N. crassa* Ca^{2+} signaling knockout mutants to the *ras-1*^{bd} mutant of opposite mating type and isolated progeny carrying the knockout mutation for the respective Ca^{2+} signaling genes in the *ras-1*^{bd} background for visualization of circadian conidiation in race tubes (Table S1 and Supplementary Method).

Determination of the period length, temperature compensation, and real-time studies

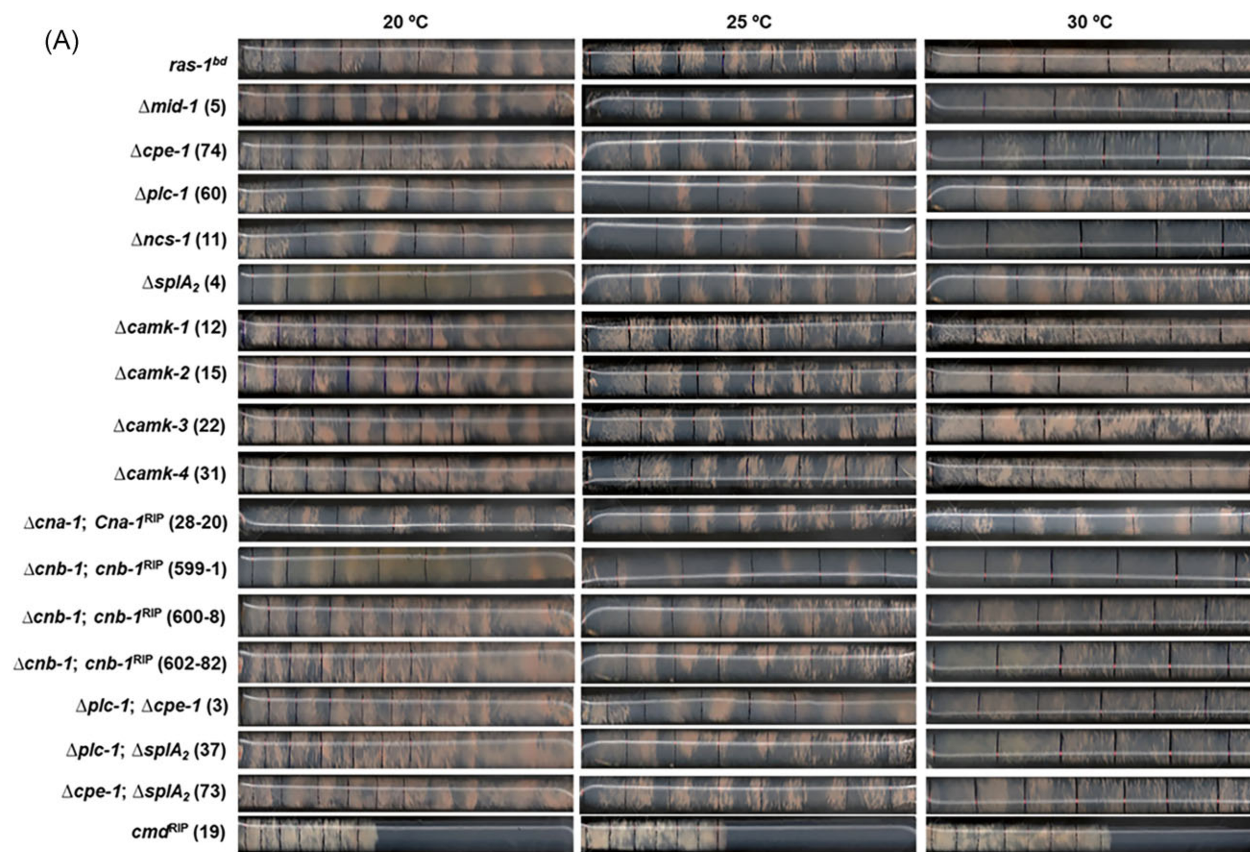
The medium containing 1X Vogel's salts, 0.17% L-arginine, 0.1% D-glucose, 50 ng/mL biotin, and 1.5% Bacto agar was used for the circadian conidiation assays in race tubes (Park and Lee 2004). To determine the period length, *N. crassa* strains were inoculated on one end of race tubes, incubated at 20, 25, or 30 °C for 24 h under constant light, and then shifted to constant darkness. The growth front was marked once per day for 7 days under red safe light. The tubes were then moved to white light, and the position of the conidial bands was marked. Period lengths were calculated by multiplying the distance between conidial bands by the inverse slope of growth front versus time (http://www.fgsc.net/teaching/circad.htm). The Q_{10} value was calculated using the formula: $Q10 = \left(\frac{R2}{R1}\right) 10/(T2 - T1)$, where R_1 and R_2 are the frequencies of the period lengths (24/Period) at T_1 and T_2 temperatures, respectively (Lakin-Thomas 1998, Sorek and Levy 2012).

For real-time studies of RNA levels, $\sim 1 \times 10^7$ conidia from the 48 h plate cultures were inoculated in flasks containing 25 ml of medium (without agar) used for the circadian conidiation assays (described above) and cultured at 125 rpm on rotary shakers in light at 20 or 25 °C (as indicated) for 2 h and then transferred to dark conditions (Aronson et al. 1994b). The mycelia were harvested after 14 h in the dark, RNA was isolated and quantitative Reverse-Transcriptase PCR (qRT-PCR) was performed (Gohain and Tamuli 2019) to determine the expression of the *frq*, *wc-1*, and *β-tubulin* genes using the primer pairs, RT-FRQ-F and RT-FRQ-R, RT-WC-1-F and RT-WC-1-R, and q-B-tub-FW and q-B-tub-RV, respectively.

Results

Multiple calcium signaling genes play a role in maintaining normal period length in *N. crassa*

We determined the period lengths in the knockout mutants of *N. crassa* Ca^{2+} signaling genes and the *ras-1*^{bd} control strain (Table 1 and Fig. 1). We determined the period length at three different temperatures (20, 25, and 30 °C) to test if the period length shows temperature compensation over a physiological range of temperatures. The *ras-1*^{bd} strain, known as the *band* (*bd*) mutant, has a T79I point mutation in *ras-1* and showed a period length of ~22.4 h at 25 °C (Belden et al. 2007). The period length of the clock in the *ras-1*^{bd} control strain was greater at 20 °C compared to 25 and 30 °C (Table 1 and Fig. 1; Gardner and Feldman 1981). We observed significantly longer periods for the Δ*camk-1*, 2, 3, and 4 mutants, particularly at 25 and 30 °C, relative to the *ras-1*^{bd} control (Table 1 and Fig. 1).



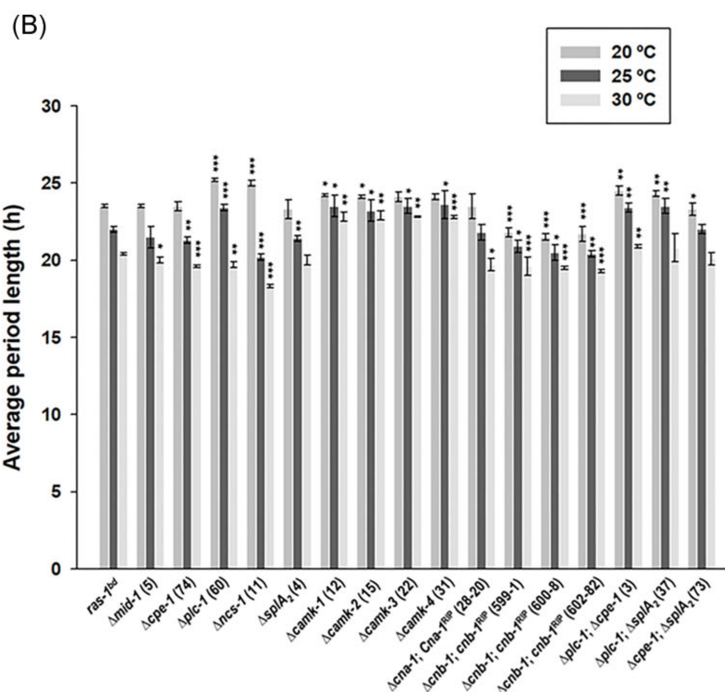


Figure 1. Circadian period and period length in the *N. crassa* Ca^{2+} signaling mutant strains. (A) Circadian-regulated conidiation in *N. crassa*. The indicated *N. crassa* strains (Table S1) were assayed for circadian-regulated conidiation at 20, 25, and 30°C using race tubes. The black lines show the growth front, marked every 24 h. The orange growth indicates the location of conidial bands. (B) Period lengths in *N. crassa* strains at 20, 25, and 30°C. Strains were inoculated on race tubes, and the cultures were incubated at 25°C in constant light for 24 h and then incubated at 20, 25, and 30°C under constant darkness. Period lengths were calculated by multiplying the distance between conidial bands by the inverse of the slope of the growth rate. Error bars show SDs calculated from the data for three independent experiments ($n = 3$) with P values < 0.05 (*), < 0.01 (**), and < 0.001 (***) relative to the *ras-1^{bd}* strain as measured by a one-way ANOVA test.

Table 1. The period length of Ca^{2+} signaling mutants at different temperatures.

Strain name	Period length (h)+		
	20 °C	25 °C	30 °C
ras-1^{bd} (Control)	23.5 ± 0.1	22.0 ± 0.2	20.4 ± 0.1
Δ*mid-1* (5)	23.5 ± 0.1	21.5 ± 0.7	20.0 ± 0.2 (*)
Δ*cpe-1* (74)	23.5 ± 0.3	21.3 ± 0.2 (**)	19.6 ± 0.1 (***)
Δ*plc-1* (60)	25.2 ± 0.1 (***)	23.4 ± 0.2 (***)	19.7 ± 0.2 (**)
Δ*ncs-1* (11)	25.0 ± 0.2 (***)	20.2 ± 0.2 (***)	18.3 ± 0.1 (***)
Δ*splA*$_2$ (4)	23.3 ± 0.6	21.4 ± 0.2 (**)	20.0 ± 0.3
Δ*camk-1* (12)	24.2 ± 0.1 (*)	23.5 ± 0.7 (*)	22.8 ± 0.3 (**)
Δcamk-2 (15)	24.1 ± 0.1 (*)	23.2 ± 0.7 (*)	22.9 ± 0.3 (**)
Δ*camk-3* (22)	24.1 ± 0.3	23.5 ± 0.5 (*)	22.8 ± 0.04 (**)
Δ*camk-4* (31)	24.1 ± 0.2	23.6 ± 0.9 (*)	22.8 ± 0.1 (***)
Δ*cna-1*; *Cna-1*^{RIP} (28–20)	23.5 ± 0.8	21.8 ± 0.5	19.7 ± 0.4 (*)
Δ*cnb-1*; *cnb-1*^{RIP} (599–1)	21.8 ± 0.3 (***)	20.9 ± 0.4 (*)	19.6 ± 0.6 (***)
Δ*cnb-1*; *cnb-1*^{RIP} (600–8)	21.5 ± 0.2 (***)	20.5 ± 0.5 (*)	19.5 ± 0.1 (***)
Δ*cnb-1*; *cnb-1*^{RIP} (602–82)	21.7 ± 0.5 (***)	20.4 ± 0.2 (**)	19.3 ± 0.1 (***)
Δ*plc-1*; Δ*cpe-1* (3)	24.5 ± 0.3 (**)	23.4 ± 0.3 (**)	20.9 ± 0.1 (**)
Δ*plc-1*; Δ*splA*$_2$ (37)	24.3 ± 0.2 (**)	23.5 ± 0.5 (**)	20.8 ± 0.9
Δ*cpe-1*; Δ*splA*$_2$ (73)	23.3 ± 0.4 (*)	22.0 ± 0.3	20.1 ± 0.4
cmd^{RIP} (19)	Not determined	Not determined	Not determined

+Results are shown as mean ± SD for three independent experiments ($n = 3$) with *P values* < 0.05 (*), < 0.01 (**), and < 0.001 (***) compared with the *ras-1*^{bd} strain as measured by a one-way ANOVA test.

The Δ*cpe-1* mutant had a slightly shorter period length than the *ras-1*^{bd} control at 25 and 30 °C. The Δ *plc-1* mutant showed period lengthening at 20 and 25 °C, and slight shortening at 30 °C relative to the *ras-1*^{bd} strain. The Δ*splA*$_2$ mutant did not show significant changes in period length at either of the temperatures tested. Because *plc-1* genetically interacts with *cpe-1* and *splA*$_2$ in *N. crassa* (Barman and Tamuli 2017), we tested the effect of this interaction on the period length. The Δ*plc-1*; Δ*cpe-1* and Δ*plc-1*; Δ*splA*$_2$ double mutants showed longer periods at 20 and 25 °C relative to the *ras-1*^{bd} control; however, the Δ*cpe-1*; Δ*splA*$_2$ double mutant did not exhibit any change in period length (Table 1 and Fig. 1). These results suggested that the *plc-1* gene is epistatic to both *cpe-1* and *splA*$_2$ for the period length at 20 and 25 °C.

The Δ*ncs-1* mutant displayed a longer period at 20 °C, and a period shortening at 25 and 30 °C (Table 1 and Fig. 1). The *cnb-1*^{RIP} mutants (Strain #599, 600, and 602; Table S1) exhibited shorter periods at all temperatures (Table 1 and Fig. 1). However, the *Cna-1*^{RIP} did not exhibit a significant change in the period length (Table 1 and Fig. 1). Because the *cmd*^{RIP} mutant (Strain #19; Table S1) showed severe growth retardation, period length could not be determined in this mutant.

Loss of certain calcium signaling genes influences temperature compensation in *N. crassa*

The Q_{10} value, which reflects the ratio of period lengths relative to a 10 °C rise in temperature, is calculated using the formula $Q10 = \left(\frac{R2}{R1}\right) 10/(T2 - T1)$, where R_1 and R_2 are the frequencies of period lengths at temperatures T_1 and T_2, respectively (Lakin-Thomas 1998, Sorek and Levy 2012). We used this equation to calculate Q_{10} values for each strain, using all combinations of period lengths at 20, 25, and 30 °C (Table 2).

The Q_{10} value ranges from 0.8 to 1.2 for normal circadian rhythms (Mattern et al. 1982, Sorek and Levy 2012). The Q_{10} value ranged from 0.8 to 1.2 for the strains lacking *cpe-1*, *splA*$_2$, *camk-1*, *camk-2*, *camk-3*, *camk-4*, and *cnb-1*, as well as for the *Cna-1*^{RIP} mutant, indicating that the circadian clock was temperature compensated in these mutants (Table 2). However, the Q_{10} value was 1.41 for the Δ*plc-1* mutant between 25 and 30 °C, and the Q_{10} value for the Δ*ncs-1* mutant was 1.53 and 1.40 when comparing 20 and 25 °C and 20 and 30 °C, suggesting a partial loss of temperature compensation of the circadian clock in these two mutants in these temperature ranges (Table 2).

Transcription of *frq* and *wc-1* was altered in certain calcium signaling mutants that displayed variation in period length

The *N. crassa* circadian clock is regulated through the interaction of three major genes *frq*, *wc-1*, and *wc-2* (Aronson et al. 1994a, 1994b, Crosthwaite et al. 1997). Differences in period length are often associated with the transcription of *frq* (Aronson et al. 1994a). In the nucleus, FRQ regulates the expression of its activators *wc-1* and *wc-2* (Cheng et al. 2001).

We performed quantitative Real-Time PCR (qRT-PCR) to measure the expression of the *frq* and *wc-1* genes at 20 and 25 °C in the strains showing period length and temperature compensation phenotypes. Transcript levels of *frq* and *wc-1* were significantly increased at 20 °C and marginally at 25 °C in the Δ *plc-1* single and Δ*plc-1*; Δ*cpe-1* and Δ*plc-1*; Δ*splA*$_2$ double mutants, temperatures at which these strains also had longer periods than the control (Fig. 2A). The difference in *frq* expression levels was most striking, with more than a 2-fold increase in the Δ*ncs-1* mutant at 20 °C, but slightly reduced expression at 25 °C (Fig. 2B, upper panel). In contrast, *wc-1* transcript levels were normal in the Δ*ncs-1* mutant at both temperatures (Fig. 2B, lower panel). Thus, there was a correlation between *frq* expression and period length in the Δ*ncs-1* mutant; this strain had a longer period at 20 °C but a shorter period at 25 °C, relative to the control. Although the strains lacking the Ca^{2+}/CaM dependent kinase genes *camk-1*, *camk-2*, *camk-3*, and *camk-4* had longer periods, we did not observe a significant difference in the *frq* and *wc-1* transcript levels in these mutants relative to the control (Fig. 2C).

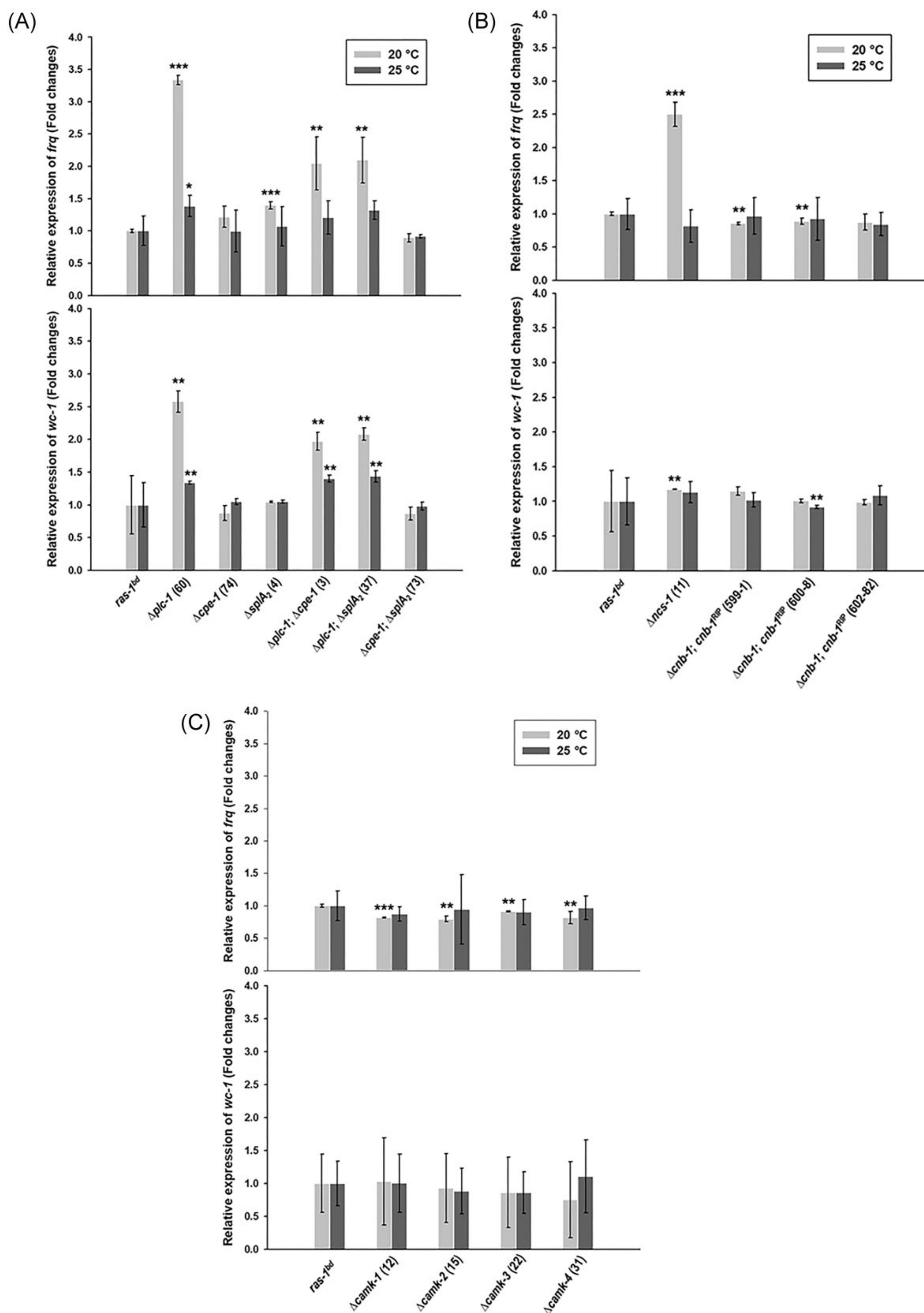


Figure 2. Expression of *frequency* (*frq*) and *white collar-1* (*wc-1*) during circadian-regulated conidiation at 20 and 25°C. RNA was isolated from the indicated groups of strains (A, B, and C) cultured under circadian-regulated conidiation conditions at 20 or 25°C and the expression of the *frq* (upper panels) and *wc-1* (lower panels) genes were determined using qRT-PCR with three biological replicates for each strain. The relative expression of each gene was normalized to the expression of the β-*tubulin* gene, and expression values were compared with those in the *ras-1*^{bd} control strain. Error bars indicate SDs calculated from the data for three independent experiments ($n = 3$) with *P* values < 0.05 (*), < 0.01 (**), and < 0.001 (***) relative to the *ras-1*^{bd} strain as measured by a one-way ANOVA test.

Table 2. Q_{10} values of the Ca^{2+} signaling mutants.

Strain name	Q_{10} values[+, ^]		
	T_1 = 20 °C, T_2 = 25 °C ($P_{20°C}$ and $P_{25°C}$)	T_1 = 25 °C, T_2 = 30 °C ($P_{25°C}$ and $P_{30°C}$)	T_1 = 20 °C, T_2 = 30 °C ($P_{20°C}$ and $P_{30°C}$)
ras-1^bd (Control)	1.14(23.5 and 22.0)	1.16(22.0 and 20.4)	1.15(23.5 and 20.4)
Δ*mid-1* (5)	1.19(23.5 and 21.5)	1.16(21.5 and 20.0)	1.17(23.5 and 20.0)
Δ*cpe-1* (74)	1.22(23.5 and 21.3)	1.18(21.3 and 19.6)	1.20(23.5 and 19.6)
Δ*plc-1* (60)	1.16(25.2 and 23.4)	1.41(23.4 and 19.7)	1.28(25.2 and 19.7)
Δ*ncs-1* (11)	1.53(25.0 and 20.2)	1.22(20.2 and 18.3)	1.40(25.0 and 18.3)
Δ*splA₂* (4)	1.18(23.3 and 21.4)	1.14(21.4 and 20.0)	1.20(23.3 and 20.0)
Δ*camk-1* (12)	1.06(24.2 and 23.5)	1.06(23.5 and 22.8)	1.06(24.2 and 22.8)
Δ*camk-2* (15)	1.08(24.1 and 23.2)	1.02(23.2 and 22.9)	1.05(24.1 and 22.9)
Δ*camk-3* (22)	1.05(24.1 and 23.5)	1.06(23.5 and 22.8)	1.06(24.1 and 22.8)
Δ*camk-4* (31)	1.04(24.1 and 23.6)	1.07(23.6 and 22.8)	1.06(24.1 and 22.8)
Δ*cna-1*; *Cna-1^RIP* (28–20)	1.16(23.5 and 21.8)	1.22(21.8 and 19.7)	1.19(23.5 and 19.7)
Δ*cnb-1*; *cnb-1^RIP* (599–1)	1.09(21.8 and 20.9)	1.14(20.9 and 19.6)	1.11(21.8 and 19.6)
Δ*cnb-1*; *cnb-1^RIP* (600–8)	1.10(21.5 and 20.5)	1.10(20.5 and 19.5)	1.10(21.5 and 19.5)
Δ*cnb-1*; *cnb-1^RIP* (602–82)	1.13(21.7 and 20.4)	1.12(20.4 and 19.3)	1.12(21.7 and 19.3)
Δ*plc-1*; Δ*cpe-1* (3)	1.10(24.5 and 23.4)	1.25(23.4 and 20.9)	1.17(24.5 and 20.9)
Δ*plc-1*; Δ*splA₂* (37)	1.07(24.3 and 23.5)	1.28(23.5 and 20.8)	1.17(24.3 and 20.8)
Δ*cpe-1*; Δ*splA₂* (73)	1.12(23.3 and 22.0)	1.20(22.0 and 20.1)	1.16(23.3 and 20.1)
cmd^RIP (19)	Notdetermined	Notdetermined	Notdetermined

[+]The related period lengths for the calculated Q_{10} values are given in parentheses.
[^]Q_{10} values marked in bold suggest partial loss of temperature compensation.

Discussion

We investigated the circadian-regulated period length for several *N. crassa* Ca^{2+} signaling mutants at three different temperatures (20, 25, and 30 °C). The Δ *camk-1*, 2, 3, and 4 mutants showed period lengthening (Table 1 and Fig. 1). A longer period phenotype has been previously reported for a Δ*camk-1* mutant strain (Yang et al. 2001). The Δ*cpe-1* mutant displayed a slightly shorter period length than the control at 25 and 30 °C. The Δ *plc-1* mutant showed a slight period shortening at 30 °C. However, the Δ *plc-1* mutant showed significant period lengthening at 20 and 25 °C. The Δ *plc-1*; Δ*cpe-1* and Δ*plc-1*; Δ*splA₂* double mutants displayed longer periods at 20 and 25 °C; however, period length in the Δ *cpe-1*; Δ*splA₂* double mutant was like the *ras-1^bd* control (Table 1 and Fig. 1). These results suggested that *plc-1* genetically interacts with *cpe-1* and *splA₂* to regulate circadian period length in *N. crassa*. Previously, the genetic interactions of *plc-1*, *cpe-1*, and *splA₂* were also found to regulate asexual and sexual developments in *N. crassa* (Barman and Tamuli 2017). In addition, the Q_{10} value was 1.41 for the Δ*plc-1* mutant (T_1 = 25 °C, T_2 = 30 °C), and 1.53 and 1.40 for the Δ *ncs-1* mutant (T_1 = 20 °C, T_2 = 25 °C; and T_1 = 20 °C, T_2 = 30 °C), suggesting a partial loss of temperature compensation of circadian clock in these mutants under the temperature conditions tested (Table 2). In addition to an increased Q_{10} value, the Δ*plc-1* and Δ*ncs-1* mutants also appeared to have an increased growth rate, as evident from the daily markings on the race tubes (Fig. 1A), and this could be due to difference in the media composition and the conditions used for the circadian-regulated conidiation assays compared to the routine cultures using VM (described in the "Materials and Methods" section). However, Δ *ncs-1* displays a slow growth phenotype (Deka et al. 2011), and Δ*plc-1* grows like the wild type (Barman and Tamuli 2015) when standard VM and growth conditions are used. In *N. crassa*, *frq⁷*, a long-period mutant, also showed an increased period and a larger Q_{10} value with partial loss of temperature compensation (Gardner and Feldman 1981, Ruoff et al. 2005).

We also determined the expression of the circadian regulators *frq* and *wc-1* under two different temperatures. The transcript levels of the *frq* and *wc-1* genes were increased >2-fold in the Δ*plc-1* single, and Δ*plc-1*; Δ*cpe-1* and Δ*plc-1*; Δ*splA₂* double mutants at 20 °C (Fig. 2A). The membrane-bound phosphoinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), inducing the release of intracellular Ca^{2+} and activation of protein kinase C (PKC), respectively (Rhee and Bae 1997). The *N. crassa* PKC is a regulator of light-responsive genes (Arpaia et al. 1999), and most of the light responses are regulated by modulating the blue light photoreceptor WC-1 (Franchi et al. 2005). In addition, PKC phosphorylates WC-1 *in vitro* (Franchi et al. 2005), and hyperphosphorylated WC-1 cannot bind to the *frq* promoter to drive its transcription (He and Liu 2005). The unphosphorylated WC-1 could efficiently bind to the *frq* promoter, leading to higher expression of *frq* mRNA (Fig. 3). Moreover, the transcription of *wc-1* is autoregulated by either light-induction or transcript stabilization processes (Ballario et al. 1996). Moreover, activation of PKC significantly decreases both *frq* and *wc-1* at the transcriptional and protein levels (Franchi et al. 2005), and endogenous DAG levels in *N. crassa* show circadian oscillation (Ramsdale and Lakin-Thomas 2000). These results suggested that loss of PLC-1 might lower DAG levels, causing PKC to remain in an inactive state that cannot phosphorylate WC-1; this could be a possible mechanism of increased expression of *frq* in the Δ*plc-1* mutant compared to the *ras-1^bd* strain (Fig. 2A, upper panel).

The above model is supported by the longer period length and higher expression of *frq* and *wc-1* mRNA observed in the Δ*plc-1* single and Δ*plc-1*; Δ*cpe-1* and Δ*plc-1*; Δ*splA₂* double mutants, particularly at 20 °C. The *cpe-1* gene encodes for a Ca^{2+}/H^+ exchanger, and this family of proteins family plays a role in controlling the resting level of $[Ca^{2+}]_c$, by transporting Ca^{2+} out of the cells and into intracellular Ca^{2+} stores in exchange for movement of H^+ ions across membranes (Zelter et al. 2004, Tamuli et al. 2013). In addition, the $sPLA_2$ enzyme catalyzes Ca^{2+}-dependent hydroly-

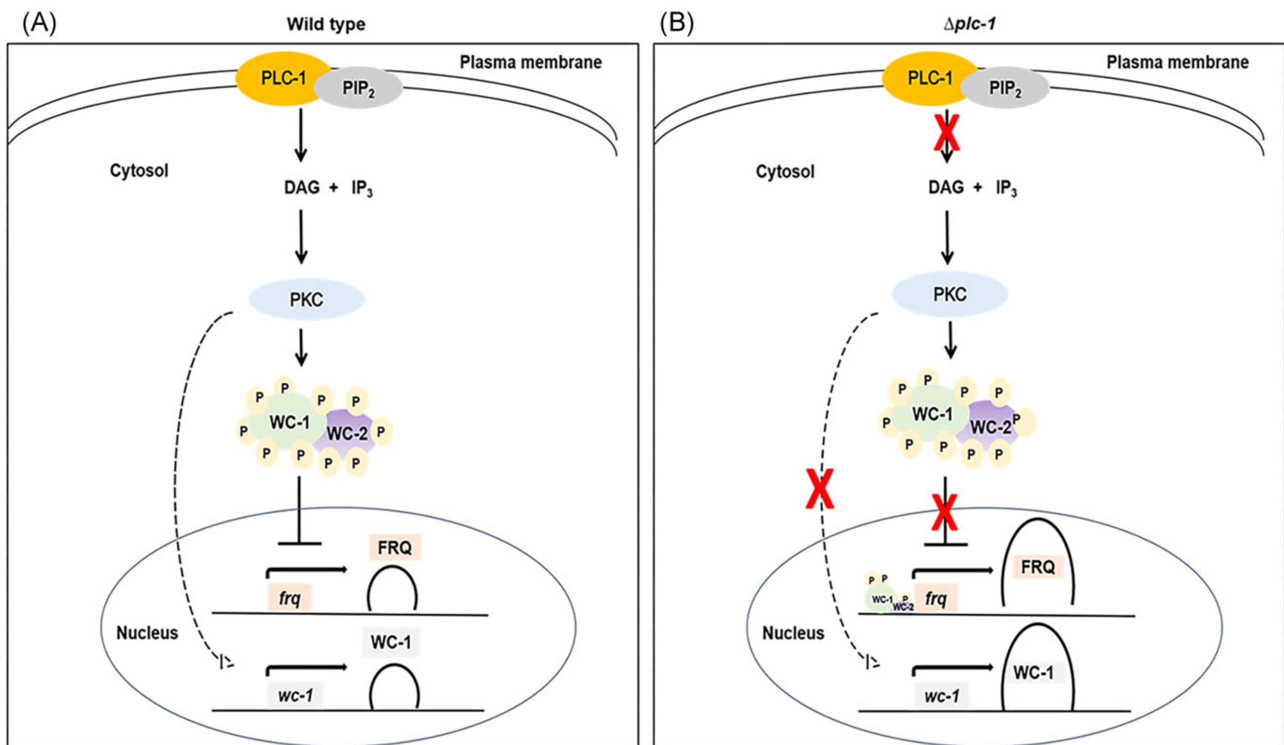


Figure 3. Model showing the role of PLC-1 in circadian-regulated conidiation. (A) PLC-1 regulates FRQ and WC-1 in the wild type strain. PLC-1 hydrolyzes PIP_2 to produce two essential second messengers, IP_3 and DAG. IP_3 causes Ca^{2+} release from the intracellular stores, and DAG activates PKC (Rhee and Bae 1997). PKC modulates the blue light photoreceptor WC-1 to control the light-responsive genes in *N. crassa* (Arpaia et al. 1999), and *frq* expression (Franchi et al. 2005). Down-regulation of PKC abolishes its effect on WC-1, causing increased *wc-1* mRNA expression and enhanced stability of the WC-1 protein (Franchi et al. 2005). In addition, the transcription of *wc-1* is autoregulated (Ballario et al. 1996). When activated, PKC interacts with the WCC complex and phosphorylates it. The hyperphosphorylated WCC complex cannot bind to the *frq* promoter to drive its expression (Aronson et al. 1994b, Wang et al. 2019), but a fall of the FRQ protein below a critical level reactivates *frq* transcription (Garceau et al. 1997, Liu and Bell-Pedersen 2006). FRQ also positively regulates the WC-1 protein level (Cheng et al. 2001). These mechanisms are essential for the normal circadian clock in *N. crassa*. (B) The effect of the *plc-1* deletion mutation on the regulation of FRQ and WC-1. We propose that the deletion of *plc-1* may negatively affect DAG levels, causing PKC to remain in an inactive state. Inactive PKC cannot phosphorylate the WCC complex, resulting in increased expression of *frq* and *wc-1* in the Δ*plc-1* single and double mutants compared to the wild type control strain.

sis of the sn_2 ester linkage of glycerophospholipids to release free fatty acids (FFAs) and 1-acyl-lysophospholipid (1-acyl-LPL), that can both act as potential signaling molecules to regulate various biological functions (Dennis et al. 2011).

We observed that the *frq* transcript level in the Δ*ncs-1* mutant was increased >2-fold relative to the *ras-1^{bd}* at 20 °C. NCS-1 responds to the increased intracellular Ca^{2+} levels and plays a role in maintaining Ca^{2+} homeostasis and tolerance to high concentrations of Ca^{2+} (Deka et al. 2011, Gohain and Tamuli 2019). Environmental stress, including low temperature, can result in increased intracellular Ca^{2+} levels (Chinnusamy et al. 2007). In addition, the increase of intracellular Ca^{2+} due to the loss of NCA-2 causes phosphorylation of FRQ and decreased period length in *N. crassa* (Wang et al. 2021). Because 30 °C is the ambient growth temperature of *N. crassa*, 20 °C may act as a stress condition that might cause an increase in the intracellular Ca^{2+} levels. Thus, Ca^{2+} homeostasis at low temperatures might be disrupted in the Δ*ncs-1* mutant. The calcineurin pathway is activated in response to high concentrations of Ca^{2+} in *N. crassa* (Gohain and Tamuli 2019, Kumar et al. 2019, Roy and Tamuli 2022). A transient increase in Ca^{2+} level at 20 °C might activate the calcineurin pathway and cause nuclear localization of WC-1 for the upregulation of the *frq* transcript in the Δ*ncs-1* mutant, resulting in lengthening of the period length in the mutant (Table 1 and Fig. 2B). In addition, intracellular Ca^{2+} induces transcription of the circadian-related *period* 1 and 2 (*m*Per1 and *m*Per2) genes via MAP kinase pathways in mouse NIH3T3 cells (Ohhashi et al. 2002). In mammals, a Ca^{2+} flux is required for maintaining circadian rhythmicity in the hypothalamic SCN (Lundkvist et al. 2005). In *Arabidopsis thaliana* and *Nicotiana benthamiana*, cytosolic free Ca^{2+} exhibits a rhythmic oscillation that relays signals relating to the circadian clock (Dodd et al. 2005). In addition, Ca^{2+} also plays a role in the regulation of the circadian rhythm and clock gene expression in *Euglena* (Goto et al. 1985), mollusks (Khalsa et al. 1993), and insects (Harrisingh et al. 2007).

Calcineurin (CNA-1) is the only serine/threonine protein phosphatase that requires Ca^{2+}/CaM for its activity (Klee et al. 1979). In response to increased $[Ca^{2+}]_c$, Ca^{2+} binds to CaM and CNB-1, which then activate CNA-1 for dephosphorylation of target transcription factors to induce expression of target genes (Rumi-Masante et al. 2012, Roy and Tamuli 2022). CaM is required to activate protein kinases and might be involved in the signal transduction from light-perceiving components to the *N. crassa* circadian clock (Sadakane and Nakashima 1996). In *N. crassa*, CNB-1 binds to the calcineurin-dependent response element (CDRE), possibly to regulate target gene expression (Kumar et al. 2006). It is conceivable that the CNB-1^{RIP} protein also has low affinity for the *frq* promoter, causing low levels of *frq* transcript (Fig. 2B). However, no direct genetic interaction could be established between these genes and *wc-1*, as *wc-1* expression was not significantly different from the control under any condition.

The strains lacking the Ca^{2+}/CaM dependent kinase genes *camk-1*, *camk-2*, *camk-3*, and *camk-4* had longer periods, but we did not observe any change in the level of *frq* and *wc-1* transcripts in these mutants (Fig. 2C). In a previous study, Ca^{2+}/CaMK-1 was shown to phosphorylate FRQ *in vitro*, and a knockout mutant of *camk-1* was found to affect the phase, period, and phase-shifting of the *N. crassa* circadian clock (Yang et al. 2001). Moreover, certain *frq* phosphorylation sites and *camk-2* were shown to be epistatic to *nca-2* (Wang et al. 2021). Another protein, casein kinase-2 (CK-2), also directly phosphorylates FRQ and plays a role in circadian temperature compensation in *N. crassa* (Mehra et al. 2009). Our results suggested that Ca^{2+}/CaMKs are not involved in regulating the expression of the *frq* gene. The increased period length observed in these mutants might result from insufficient phosphorylation and/or a longer time to phosphorylate FRQ before it is ubiquitinated.

In conclusion, similar to the long-period mutant frq^7 (Ruoff et al. 2005), a partial loss of temperature compensation was observed in the Δ*plc-1* and Δ*ncs-1* mutants. The *frq* and *wc-1* transcript levels were increased in the Δ*plc-1*, Δ*plc-1*; Δ*cpe-1*, and Δ*plc-1*; $\Delta splA_2$ mutants at 20 °C, suggesting that *plc-1* plays a role in the circadian period length by regulating the expression of *frq* and *wc-1*. In addition, the *frq* transcript level was also increased in the Δ*ncs-1* mutant at 20 °C, suggesting that *ncs-1* regulates the *frq* transcription via a mechanism yet to be identified. Further studies will establish the detailed molecular pathways used by these Ca^{2+} signaling genes to regulate circadian period length in *N. crassa*.

Author Contribution

D.B., C.M., A.R., D.G., and A.K. performed experiments, and prepared figures and tables. D.B. also wrote the revised drafts and prepared the final figures. P.D. helped in some experiments. K.A.B. helped in generating some of the *N. crassa* strains used in this study, provided suggestions, and corrected the manuscript. RT designed experiments, wrote, and edited the manuscript.

Ethical Statement

The proper ethical standard was followed in this research.

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Supplementary Data

Supplementary data are available at *FEMSLE* online.

Conflict of Interest. The authors declare no conflict of interests.

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Data Availability

Strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present in the article, figures, and tables.

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