Characterizing Roles of Kinase *stk-16* and Phosphatase *pzl-1* in Photoperiodic Regulation in *Neurospora Crassa*

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Abstract

Photoperiodism, the biological response to changes in day length, influences reproductive processes in many organisms. In this study, we conducted research on specific genes in the model organism *Neurospora crassa*, a fungus known for its well-characterized genetic system and responsiveness to environmental cues. We developed the Protoperithecia Assay (PPA) to measure female reproductive structures in *N. crassa* that respond to different photoperiods. Phosphorylation, a critical regulatory mechanism controlled by kinases and phosphatases, is hypothesized to mediate these responses. According to prior findings from the Fungal Genetics Stock Center, the kinase mutant *stk-16* (FGSC#13072) significantly reduces protoperithecia formation, whereas the phosphatase mutant *pzl-1* (FGSC#11548) leads to increased development. We analyzed these mutants (*stk-16* and *pzl-1*) under different photoperiod conditions (short-day, long-day, and equinox) using the Protoperithecia Assay (PPA). The current study aims to validate previous observations and confirm that the data are reproducible. We also confirmed the gene knockouts of interest by PCR, which verified that the kinase is relevant to our study. Understanding these mechanisms in a model system like *N. crassa* could provide insight into broader principles of seasonal and circadian regulation in eukaryotes

Hypothesis

The kinase *stk-16* and the phosphatase *pzl-1* regulate reproductive responses to day length in *N.crassa* by mediating its adaptation to different photoperiods.

Circadian Rhythm and Photoperiodism

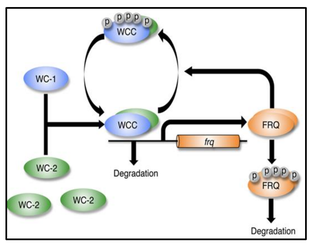

Figure 1A. Background of Circadian Rhythm. The White Collar Complex (WCC), made of WC-1 and WC-2, transcribes the *frq* gene and then translates the FRQ protein. When the FRQ protein builds up, it later blocks WCC, stopping more *frq* from being made.

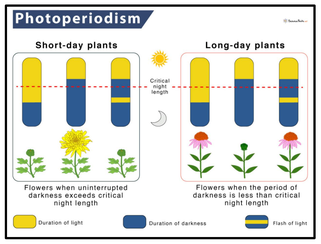

Figure 1B. Photoperiodism in plants. SD plants require nights that are longer than the critical length, and they can only flower if the darkness is uninterrupted. LD plants require nights that are shorter than the critical length, and they can flower even if the darkness is interrupted

Materials and Methods

Protoperithecia Assay (PPA) and PCR stk-16 KO

In this study, we used the FGSC 2489 (wild type), FGSC 13072 (*stk-16* mutant), FGSC 11548 (*pzl-1* mutant), and FGSC 11554 (*frq mutant)* strains of *N. crassa* in minimal media. We transferred the samples onto Petri dishes containing synthetic crossing media. The plates were placed in chambers and incubated at 25 °C for seven days. After incubation, we ensured the hyphae were cleared, took images under a microscope. Using ImageJ software, we counted protoperithecia production. We confirmed stk-16 KO by doing PCR, designing primers using SnapGene.

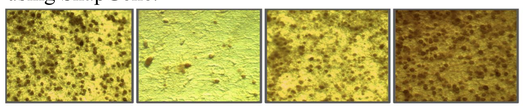

Figure 2. Assay. Under microscopic observation, it presents the development of **Protoperithecia** on petri dishes after light exposure under EQ conditions. Each visible speck marks the presence of a protoperithecia. The images include the wild-type FGSC 2489, the kinase mutant FGSC 13072, the phosphatase mutant FGSC 11548, and the *frq* mutant FGSC 11554.

Figure 3: Photoperiodic Conditions

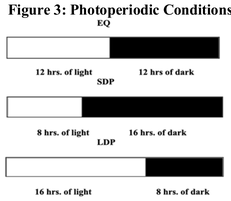

Figure 3: Photoperiodic Conditions

We used three different photoperiodic conditions: Equinox (EQ), Short-Day Photoperiod (SDP), and Long-Day Photoperiod (LDP). For EQ, cultures were exposed to 12 hours of light : 12 hours of darkness. SDP consisted of 8 hours of light and 16 hours of darkness. LDP is 16 hours of light and 8 hours of darkness

Figure 4A: Map of Oligo Position

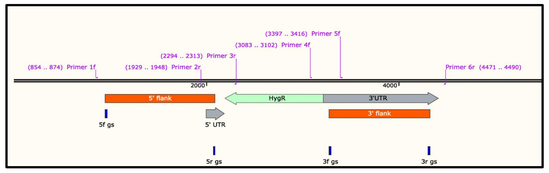

Figure 4A: Six primers were designed to confirm the replacement *stk-16* with the hygromycin resistance cassette (*HygR*). Primers 1f and 2r amplify the 5′ gene-specific region, while primer combinations (1f + 3r, 4f + 6r) span the junctions between the *HygR* cassette and the flanking regions.

Figure 4B: Table showing the expected PCR results

Primer Combination	Expected base pairs	Expected banding Wild Type	Expected *stk-16* KO
Primer (1f + 2r)	1095 bp	+	+
Primer (1f + 3r)	1460 bp	-	+
Primer (4f + 6r)	1408 bp	-	+
Primer (5f + 6r)	1094 bp	+	+

Figure 4B: Table showing the expected PCR results for the *stk-16* knockout test. The table lists the size of DNA bands in base pairs for each primer pair and whether bands are expected in wild-type or knockout samples.

Figure 4C: PCR image of *stk-16* KO and wildtype

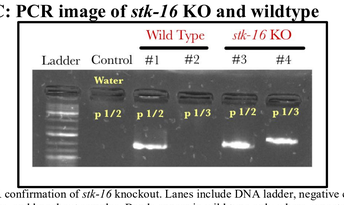

Figure 4C: PCR confirmation of *stk-16* knockout. Lanes include DNA ladder, negative control (water), wild type samples, and knockout samples. Bands appear in wild type only where expected, while knockout lanes show band patterns confirming the gene replacement. Primer pairs are labeled as P1/2 and P1/3, where P1/2 represents primer 1 + primer 2 (gene-specific site) and P1/3 represents primer 1 + primer 3.

Data

Figure 5: Protoperithecia production under Different photoperiods

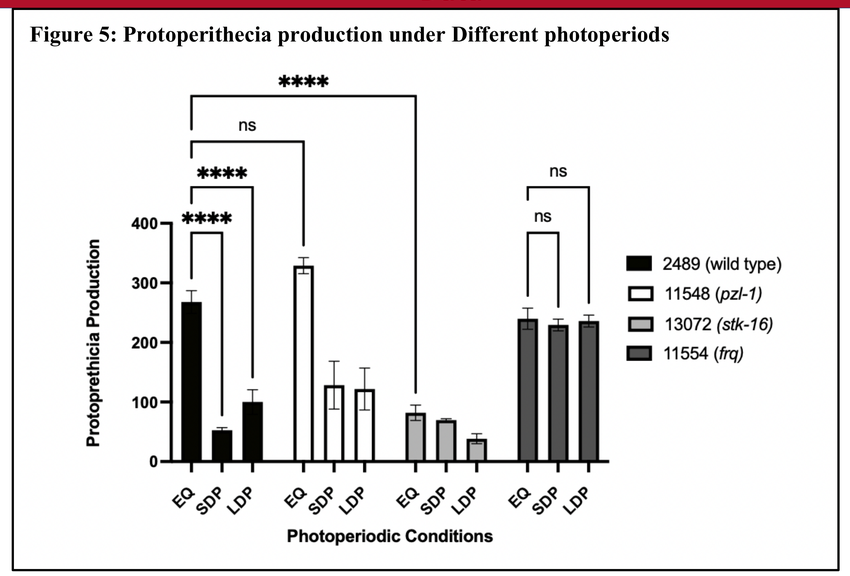

Figure 5. Protoperithecia production under different photoperiod conditions in FGSC 2489 (wild type), FGSC 11548 (*pzl-1* mutant), FGSC 13072 (*stk-16* mutant) strains, and FGSC 11554 (*frq* mutant strains).
The number of protoperithecia was quantified after 7 days of exposure to different photoperiods (EQ = equinox condition, SDP = short day period, LDP = long day period). Data are mean ± SEM (n=4). Statistical significance was determined by two-way ANOVA.

Discussion and Conclusion

To test our hypothesis that the *stk-16* kinase and *pzl-1* phosphatase contribute to photoperiodic regulation in *Neurospora crassa*, we performed PPA (Materials and Methods) under different photoperiodic conditions. As expected, the wild-type strain (FGSC 2489) exhibited a significant decrease in protoperithecia production under both short-day period (SDP) and long-day period (LDP) conditions compared to the equinox (EQ) condition ($p < 0.001$ and $p < 0.05$, respectively; Fig. 4 This variation in protoperithecia formation across day-length conditions has been used as a proxy phenotype for assessing *N. crassa*'s ability to measure day length. Previous studies have reported that the circadian clock contributes to this regulation. To confirm this, we used the *frq* mutant, which lacks the key circadian clock gene FREQUENCY, as a control. As expected, this mutant showed no difference in protoperithecia production across photoperiod conditions (Fig. 5). We reasoned that if a gene is required for day-length measurement, then mutants lacking that gene would fail to show differential protoperithecia production across photoperiods. Consistent with this prediction, the *stk-16* kinase mutant lost this differential response, whereas the *pzl-1* phosphatase mutant retained the wild-type pattern.

Based on these results, we conclude:

- *N. crassa* can measure day length and regulate female organ production in a circannual cycle.
- The circadian clock is involved in photoperiodic regulation.
- FGSC 13072 is the authentic *stk-16* knockout strain.
- STK-16 kinase is required for photoperiodic regulation.
- PZL-1 phosphatase is not involved in photoperiodic regulation.

Directions for Future Research

For future studies, we would like to investigate the details of the *stk-16* mutant to understand how this gene is involved in the molecular mechanisms of circadian rhythm

Acknowledgements

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Reference

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