THE LIGHT MUTANT OSCILLATOR (LMO): A NOVEL CIRCADIAN OSCILLATOR IN *NEUROSPORA CRASSA*

A Thesis

by

HE HUANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2008

Major Subject: Biology

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Approved by:

Chair of Committee,	Deborah Bell-Pedersen
Committee Members,	Daniel Ebbole
	Susan Golden
	Wayne Versaw
Head of Department,	Vincent Cassone

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ABSTRACT

The Light Mutant Oscillator (LMO): A Novel Circadian Oscillator in *Neurospora crassa*. (August 2008)

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Chair of Advisory Committee: Dr. Deborah Bell-Pedersen

Circadian clocks are present in most eukaryotes and some prokaryotes and control rhythms in behavior, physiology and gene expression. One well-characterized circadian clock is that of *Neurospora crassa*. In addition to the well-described *N. crassa* FRQ/WCC oscillator, several lines of evidence have implied the presence of other oscillators which may have important functions in the *N. crassa* circadian clock system. However, the molecular details are only known for the core FRQ/WCC oscillator. The light mutant oscillator (LMO) was identified by two mutations (LM-1 and LM-2) and shown to control developmental rhythms in constant light (LL), conditions in which the FRQ/WCC oscillator is not functional. The objective of this project was to determine whether the developmental rhythms driven by the LMO are circadian, whether the components of the LMO communicate with components of the FRQ/WCC oscillator, and to begin to define the molecular nature of the LMO.

First, the conditions for growth of the LM-1 mutant strain that reveals the best circadian rhythm of development in LL were found. Second, the LMO was determined to display

the three properties required of a circadian oscillator. Third, the LMO was shown to function independently of the FRQ/WCC oscillator to control developmental rhythms in LL. However, evidence suggests that the FRQ/WCC oscillator and the LMO communicate with each other. Finally, using Cleaved Amplified Polymorphic Sequence (CAPS) markers, the LM-1 mutation was genetically mapped to the right arm of linkage group I within a 1069 kb region. Together, these results provide a start towards understanding of the complexity of oscillators that form a circadian clock in organisms.

ACKNOWLEDGEMENTS

I would like to thank my committee members, Drs. Daniel Ebbole, Susan Golden, and Wayne Versaw, for their support and advice throughout the course of this research over the years. I especially thank Dr. Deborah Bell-Pedersen, whom I respect as a perfect model; her sage advice, scientific rigor and continued support always inspired me. She really put a lot of time into nurturing me and tried her best to transform me from a chemical engineering undergraduate student to a good biology graduate student. I would also like to thank the rest of the members of the Bell-Pedersen lab: Lindsay Bennett, Dr. Renato de Paula, Johnny Fazzino, Chuck Goldsmith, Dr. Andrew Greene, Dr. Teresa Lamb, Dr. Shaojie Li, Dr. Xiaoguang Liu, Dr. Louis Morgan, Imade Nsa and Dr. Mike Vitalini, for their continuous help and support. It has been a wonderful experience both personally and professionally, and you all have made the lab such a wonderful place to work. Special thanks go to Dr. Teresa Lamb for answering all my experimental questions and editing my thesis. Thanks also go to my friends and the department faculty and staff for making my time at Texas A&M University a great experience. Finally, I would like to sincerely thank my parents and my fiancée, Jingjing Xu, for their encouragement, patience and love.

NOMENCLATURE

bd	*band*
CAMK-1	calcium/calmodulin-dependent kinase-1
CAPS	cleaved amplified polymorphic sequence
C-box	clock-box
ccg	clock-controlled gene
CDO	choline deficiency oscillator
CK	casein kinase
CT	circadian time
DAG	diacylglycerol
DD	constant dark
DNA	deoxyribonucleic acid
FFC	FRQ/FRH complex
FGSC	Fungal Genetic Stock Center
FLO	FRQ-less oscillator
FRH	FRQ-interacting RNA helicase
FRP	free-running period
frq	*frequency*
FWD-1	F-box/WD-40 repeat-containing protein-1
GFP	green fluorescent protein
kb	kilobases

LD	light/dark
LL	constant light
LM	light mutant
LMO	light mutant oscillator
Ma	Mauriceville
mRNA	messenger ribonucleic acid
NHEJ	non-homologous end-joining
NRA	nitrate reductase activity
NRO	nitrate reductase oscillator
OR	Oak Ridge
ORF	open reading frame
PAS	PER-ARNT-SIM
PCR	polymerase chain reaction
qde-1	*quelling-defective-1*
RFP	red fluorescent protein
SCN	suprachiasmatic nucleus
TC-FLO	temperature controlled – FLO
UV	ultraviolet light
wc	*white collar*
WT	wild type

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1. INTRODUCTION

1.1 Circadian Clocks

Circadian clocks are endogenous molecular timekeepers consisting of one or more oscillators. These clocks are present in most eukaryotes and some prokaryotes (reviewed by Young and Kay, 2001). The clock imparts temporal organization on a variety of cellular processes, ranging from development in fungi, photosynthesis in plants, sleep/wake cycles in animals, to cognitive functions in humans (reviewed by Bell-Pedersen et al., 2005). Circadian clocks allow organisms to anticipate and prepare for predictable daily rhythms in the environment that take place as a consequence of the earth's rotation on its axis. Linking the internal timing mechanism to external cycles allows organisms to optimize their survival and reproductive success (reviewed by Dunlap et al., 2004).

Because circadian clocks in humans control many aspects of physiology and gene expression, it is not surprising that disruptions of normal circadian rhythms are associated with disease. People who carry out shift work or who have a defective clock are at increased risk for depression, obesity, headaches, cardiovascular disease, epilepsy, cerebrovascular malfunction and cancer (Lei et al., 2006; reviewed by Turek et al., 2001). However, the precise mechanisms for the link between the clock and disease are

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not known. An understanding of circadian clock mechanisms and their underlying complexity may lead to novel treatments for disease and conditions linked to circadian pathologies.

Circadian rhythms are defined by three key characteristics (reviewed by Dunlap et al., 2004).

(1) Circadian rhythms persist with an intrinsic free-running period (FRP) of roughly 24 h, but not usually exactly equal to 24 h, when the organism is kept under constant conditions, such as constant light (LL) or constant dark (DD) and temperature. The FRP is dependent on a variety of factors, including organism species, developmental factors, ambient temperature, illumination and prior history.

(2) Circadian rhythms can be entrained by external cues, including temperature or light, in a time-dependent fashion. One of the most common external inputs is the light/dark (LD) cycles generated by the rising and setting of the sun. Entrainment synchronizes the endogenous oscillators to local time, enabling organisms to anticipate and prepare for physical environmental changes and to organize their activities to the appropriate times of day.

(3) Circadian rhythms are temperature-compensated, meaning the FRP of circadian rhythms are nearly unaffected across an extensive range of physiologically relevant

temperatures. The effect of temperature changes on the rate of biological processes is determined by their Q_{10} values (Pittendrigh, 1954). Q_{10} is a gauge of the temperature dependency of a process, and it is calculated as the ratio of the rate of a process at a higher temperature divided by the rate at a temperature 10 °C lower. If the Q_{10} value of a process is close to 1, then the rate is essentially temperature-independent. The Q_{10} values for most enzymatic reactions are 2 or 3, while the Q_{10} values for circadian rhythms range from 0.8 to 1.4, when calculated within the physiological range for the organisms (Sweeney and Hastings, 1960). For *Neurospora crassa*, the Q_{10} value for the developmental rhythm in DD is 1.03 (Sweeney et al., 1960). Temperature-compensation is believed to be just one facet of the general compensation machinery in cells which maintains constant period length despite differences in parameters influencing metabolism, such as nutrition or temperature.

1.2 The Molecular Basis of Circadian Clocks

As illustrated in Figure 1, a simplified circadian clock system is composed of three main parts (reviewed by Bell-Pedersen et al., 2005). The input pathways transduce external cues to the clock for entrainment. One or more oscillators, the time pieces of the clock system, receive signals from the input pathways, create a program with the length of about 24 h, and in turn transfer phase information via the output pathways to temporally regulate molecular, biochemical, physiological and behavioral processes.

As demonstrated in Figure 2, eukaryotic circadian oscillators consist of positive and

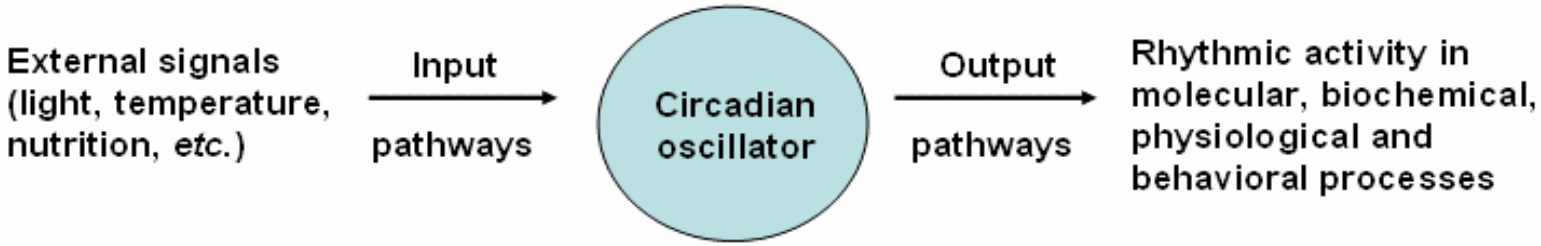


Figure 1. A Simplified View of the Circadian Clock System.
See the text for details of the figure. Arrows represent the flow of information.

negative components that form auto-regulatory feedback circuits (reviewed by Harmer et al., 2001; Bell-Pedersen et al., 2005). In these loops, positive elements transcriptionally activate the "clock genes" which encode the negative elements. As a result, the concentrations of the negative elements increase. The negative elements repress the activities of the positive elements. This repression inhibits the transcription of genes encoding the negative elements. Phosphorylation-induced degradation of the negative elements reduces their concentrations, which results in the reactivation of positive elements and the start of a new cycle the next day. In eukaryotes, the negative elements also trigger the expression of one or more of the positive elements to generate interlocking positive and negative feedback loops, which are critical for maintaining the precision of the clock (reviewed by Liu and Bell-Pedersen, 2006). All of these events impose temporal delays within the central feedback circuit, such that the molecular cycle takes about 24 h to be finished. Components of the oscillators signal time of day information through output pathways to regulate rhythmic gene expression and overt rhythmicity. While great progress has been made in understanding the molecular basis of circadian rhythmicity, accumulating evidence suggests that a single oscillator is not sufficient to control all rhythmicity in organisms. For example, two rhythms that run simultaneously with different periods have been demonstrated in Gonyaulax and rat (Morse et al., 1994; Cambras et al., 2007), and residual rhythmicity has been observed in strains that are defective in core oscillator components (Loros and Feldman, 1986; Correa et al., 2003; de Paula et al., 2006; Stanewsky et al., 1998; Emery et al., 2000; Collins et al., 2005). In higher eukaryotes, some peripheral tissue-specific oscillators are

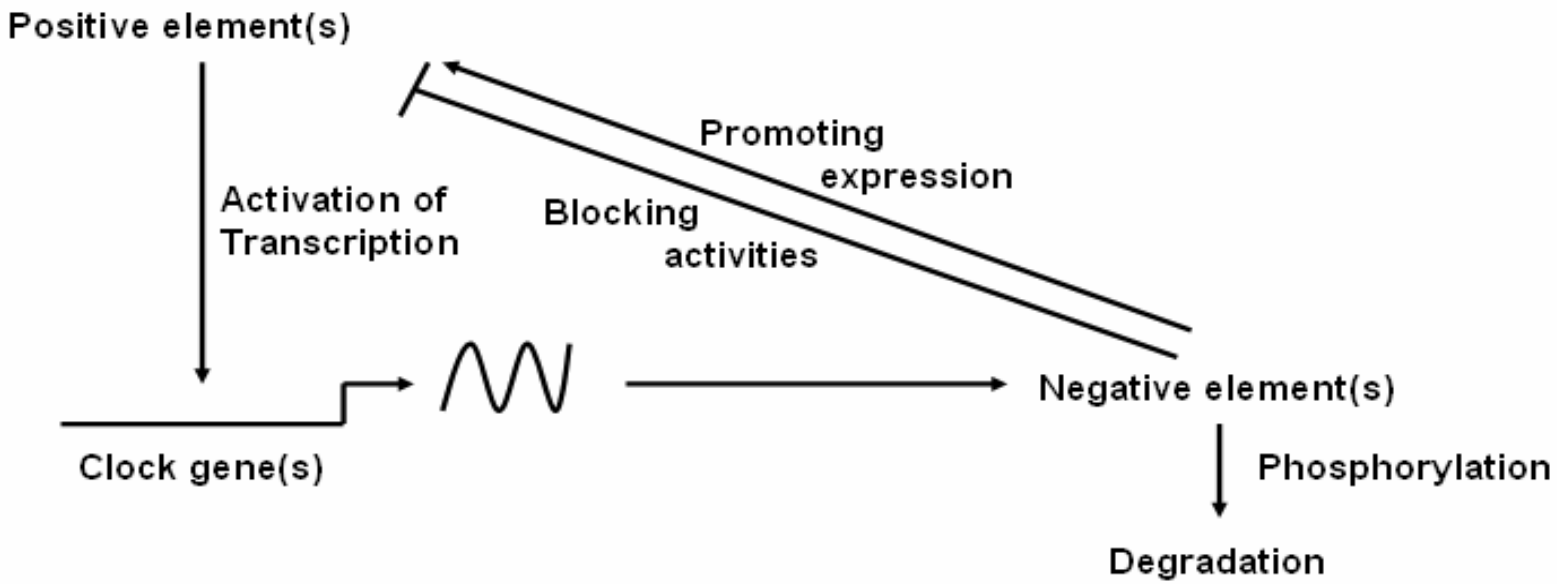


Figure 2. Common Molecular/Biochemical Themes in Eukaryotic Circadian Oscillators.
See the text for details of the model. ∿ represents rhythmic mRNA, → represents activation, ⊣ represents repression and ⌐→ represents the promoter of clock gene(s).

built differently from oscillators in the brain (Collins et al., 2005; Ivanchenko et al., 2001; Krishnan et al., 2001; Hardin, 2003). Despite this evidence for the existence of multiple oscillators, the molecular details of these additional oscillators are not yet known.

1.3 The *Neurospora crassa* Clock

Studying the circadian clock system of the filamentous fungus *N. crassa* has uncovered many of the basic principles which underlie circadian rhythms, such as negative feedback and temperature and light entrainment (reviewed by Liu and Bell-Pedersen, 2006, Heintzen and Liu, 2006, Liu 2003a and Loros and Dunlap, 2001).

The clock in *N. crassa* controls an easily monitored circadian rhythm in asexual spore development (conidiation). This assay was particularly helpful for investigating the effects of mutations on clock function (reviewed by Loros and Dunlap, 2001). The conidiation rhythm in WT clock strains has a period of approximately 22 h at 25°C in DD. The circadian rhythm of conidiation is usually monitored using the race tube assay (Figure 3) (Sargent et al., 1966). In this assay, conidia are inoculated at one end of 30 to 40 cm long cylindrical glass tubes that are bent upward at the ends to accommodate a solid growth medium. The race tubes are usually scanned to be documented as digital images which can be used later to generate plots representing the density of conidiation over the course of experiments. Typically, strains used for the analysis of the conidiation rhythm on race tubes contain the *band (bd*) mutation (recently determined to be a point

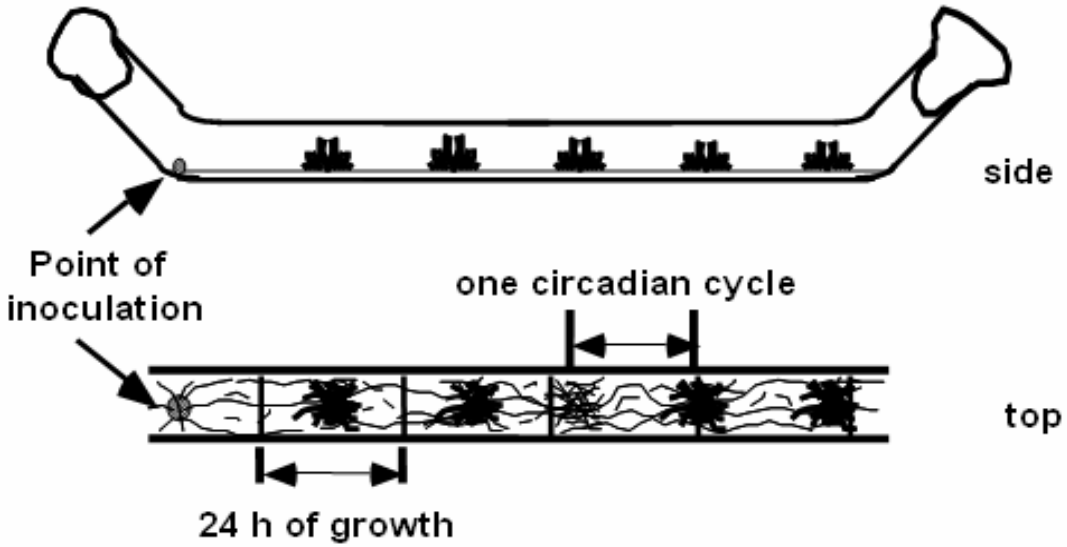


Figure 3. Cartoon Depiction of the Race Tube Assay. (redrawn from Bell-Pedersen, 2000) Conidia are inoculated at one end of a cylindrical glass tubes that are bent upward at the ends to accommodate a solid growth medium. The culture is allowed to germinate and grow in LL for about 24 h, after when the growth front is marked on the tube and the culture transferred to DD. This LL to DD transfer synchronizes the cells to dusk. The growth direction is from left to right and solid vertical lines correspond to 24 h growth. The bands are asexual developmental fluffy conidiospores. Periods of the rhythms (the time between two consecutive conidial bands, h) and growth rates (the distance of the growing front in 24 h, cm/day) can be calculated from the race tubes.

mutation in *ras-1* [Belden et al., 2007]). The *ras-1*^{bd} mutation clarifies the developmental rhythm on race tubes, and decreases the growth rate of strains, but does not alter the circadian clock. Monitoring rhythmic mRNA and protein accumulation is also routinely performed by culturing mycelia in shaking liquid medium and harvesting similar-age mycelia at different circadian times (CT) (Loros et al., 1989). CT is a formalism that is used to normalize biological time in strains or organisms with different FRPs to 24 circadian h per cycle. By convention, CT 0 is dawn, and in diurnal animals CT 0 is the beginning of the activity phase. CT 12 represents dusk, and for nocturnal animals it designates the start of the activity phase (reviewed by Dunlap et al., 2004).

Studies of the *N. crassa* clock are enhanced by the excellent genetics and biochemistry of the organism. The haploid genome of seven chromosomes has been sequenced to reveal a genome size of ~40 MB and ~10,000 genes (Borkovich et al. 2004). The use of mutations in the non-homologous end-joining (NHEJ) pathway permits near 100% recombination between two homologous DNA's and efficient gene replacement (Ninomiya et al., 2004). These mutations have been used to generate a large scale gene knockout library (Colot et al., 2006), available through the Fungal Genetic Stock Center (FGSC) (McCluskey, 2003). 70-mer oligonucleotide microarrays representing each predicted open reading frame are also available through the FGSC (Kasuga et al., 2005). A recent development in *N. crassa* is the introduction of green fluorescent protein (GFP) (Freitag et al., 2004) and red fluorescent protein (RFP) (Freitag and Selker, 2006)

fusions to visualize proteins in living cells, as well as luciferase for monitoring gene activity, including rhythmic gene expression (Lewis et al., 2002; Gooch et al., 2008).

The well-characterized FRQ/WCC oscillator was the first oscillator discovered in *N. crassa* and was long considered to be the only circadian oscillator regulating overt circadian rhythms (reviewed by Heintzen and Liu, 2006). Similar to all known eukaryotic oscillators, the FRQ/WCC oscillator is comprised of an auto-regulatory, transcriptional/translational, negative feedback loop. This loop involves the *frequency (frq), white collar-1 (wc-1)* and *wc-2* genes and their protein products, and a FRQ-interacting RNA helicase (FRH) (reviewed by Heintzen and Liu, 2006). In this feedback loop, FRQ/FRH complex (FFC) forms the negative limb of the oscillator, whereas WC-1 and WC-2, two PER-ARNT-SIM (PAS) domain-containing transcription factors, are the positive elements (Figure 4) (Aronson et al., 1994b, Cheng et al., 2005; Crosthwaite et al., 1997).

During late subjective night, in cultures grown in DD, WC-1, the main blue-light photoreceptor of *N. crassa* (He et al., 2002; Froehlich et al., 2002; Lee et al., 2003), and WC-2 proteins dimerize through their PAS domains in the nucleus forming a dark WC complex (D-WCC). The D-WWC binds to the clock box (C box) in the *frq* promoter, to directly activate the transcription of the *frq* gene (Cheng et al., 2005; Froehlich et al., 2003; He and Liu, 2005b). In the early subjective morning, *frq* mRNA levels peak, while levels of FRQ protein slowly increase and enter the nucleus. The peak in FRQ protein

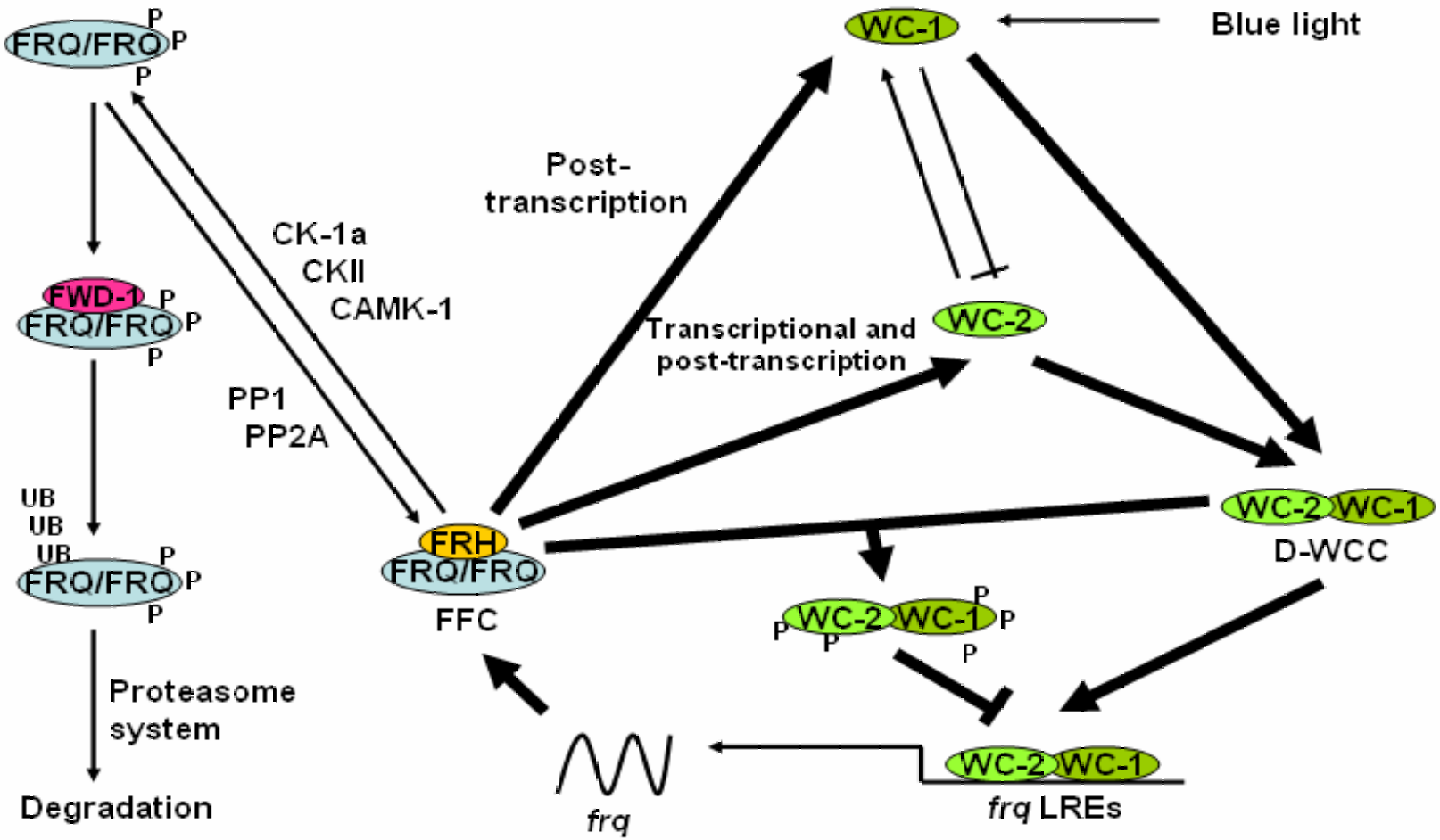


Figure 4. Model of the *N. crassa* FRQ/WCC Oscillator. (based on Heintzen and Liu, 2006)

See the text for details of the model. ∿ represents rhythmic mRNA, → represents activation, ⊣ represents repression, the thicker arrows represent the core of the oscillator, P represents phosphorylation and UB represents ubiquitination.

accumulation occurs with about a 4 to 6 h lag from the peak in *frq* mRNA in the late subjective day (Aronson et al., 1994b; Garceau et al., 1997).

After FRQ is synthesized, it self-dimerizes and forms the FFC with FRH in the nucleus, and then the FFC binds to D-WCC leading to the phosphorylation of WCC (Cheng et al., 2005; Merrow et al., 1999). Once hyperphosphorylated, activity of the D-WCC is repressed such that it is unable to transcriptionally activate *frq* (Cheng et al., 2005; Froehlich et al., 2003). This repression leads to a decrease of *frq* mRNA levels beginning around mid-subjective day, reaching a trough around mid-subjective night. Repression also leads to a reduction of FRQ protein levels, forming the negative limb of the feedback loop.

While FRQ is directing WCC phosphorylation, it is also being progressively phosphorylated over time by several kinases, including casein kinase I (CK-1a), CKII and calcium/calmodulin-dependent kinase (CAMK-1), and dephosphorylated by two phosphatases, PP1 and PP2A (Liu, 2005). After FRQ is fully phosphorylated, it physically interacts with FWD-1, an F-box/WD-40 repeat-containing protein and the substrate-recruiting subunit of an SCF-type ubiquitin ligase complex, which mediates the ubiquitination of FRQ and its degradation by the proteasome system (He et al., 2003). The loss of FRQ, in conjunction with dephosphorylation of the WCC by PP2A, releases the repression of D-WCC, allowing the cycle to restart (reviewed by Heintzen and Liu, 2006).

In addition to repressing D-WCC activity, FRQ also functions in a positive feedback loop (Cheng et al., 2003b; Merrow et al., 2001), promoting the transcription of *wc-2* and increasing the levels of WC-1 and WC-2 proteins post-transcriptionally (Lee et al., 2000; Schafmeier et al., 2006). This positive loop confers stability and robustness to the FRQ/WCC oscillator (Cheng et al., 2001). The mechanisms for this regulation are not clear; however, it was recently shown that phosphorylation of the PEST-2 region of cytoplasmic FRQ is required for its role in supporting WC-1 accumulation (Schafmeier et al., 2006). Therefore, FRQ functions in at least two roles in the positive and negative feedback loops, interlocking the repression of its own transcript with the up-regulation of the levels of the WC proteins. Progressive phosphorylation of FRQ seems to facilitate a change in FRQ from a nuclear repressor to a cytoplasmic activator of WC-1 protein accumulation (Brunner and Schafmeier, 2006). This process takes about 14 h; therefore, phosphorylation appears to be a major player in delaying the cycle.

WC-1 and WC-2 also regulate each other to form an additional interacting feedback loop in the FRQ/WCC oscillator. WC-2 supports WC-1 protein accumulation by forming the D-WCC (Cheng et al., 2002). On the other hand, WC-1 negatively regulates the expression of *wc-2* at the level of transcript abundance (Cheng et al., 2003a). This feedback regulation between *wc-1* and *wc-2* keeps D-WCC at optimal levels for its function in the both circadian rhythms and blue light sensing (reviewed by Heintzen and Liu, 2006).

1.4 Oscillator Complexity in the *Neurospora crassa* Clock System

In addition to the well-described FRQ/WCC oscillator, several lines of evidence have implied the presence of FRQ-less oscillators (FLOs) which may have important functions in the *N. crassa* circadian clock system, such as a FRQ-less oscillator (FLO), temperature controlled-FLO (TC-FLO), choline deficiency oscillator (CDO), nitrate reductase oscillator (NRO), rhythms in diacyglycerol (DAG) levels and WC-dependent FLO (WC-FLO) (Table 1). However, molecular details are only known for the core FRQ/WCC oscillator. The data supporting additional oscillators have raised awareness and rekindled an appreciation for the early work by Collin Pittendrigh in flies suggesting multiple coupled oscillators form the clock (Pittendrigh, 1954). Multiple oscillators may add stability and robustness to the clock system (Cheng et al., 2001; Locke et al., 2006; Preitner et al*.,* 2002), increase the flexibility of the system during entrainment, and allow different oscillators to control phase or functionally related outputs (Rand et al., 2006; de Paula et al., 2007).

To begin to identify components of the FLOs, we undertook a genetic screen for FRQ suppressors. About 8000 ultraviolet light (UV) mutagenized *wc-2*^{234W}*; bd;* Δ*frq* mutant colonies were screened for developmental rhythms in LL. This strain lacks FRQ and has a nonfunctional WC-2 protein; thus, this strain was chosen for its complete absence of a functional FRQ/WCC oscillator. In WT strains in LL, FRQ protein accumulates to high levels all day long, and the overt rhythm in conidiation is abolished. A developmental rhythm is also not observed in ΔFRQ strains in LL (Crosthwaite, 1995). We identified

Table 1. FRQ-less Oscillators in *N. crassa*

Name	Description	Limit
FRQ-less oscillator (FLO)	Strains lacking FRQ revealed developmental rhythms with periods from 12 to 35h depending on media and growth conditions (Aronson et al., 1994a; Loros and Feldman, 1986).	Not clear if the developmental rhythms are circadian rhythms since the rhythm has a variable period.
temperature controlled-FLO (TC-FLO)	Δ*frq* developmental rhythms can be entrained by 5°C cycles in LL or DD (Merrow et al., 1999). This rhythm was later suggested to be the effect of temperature on development (Pregueiro et al., 2005). Lakin-Thomas subsequently showed both entrained and temperature driven rhythms exist. The Δ*frq* conidiation rhythm is stablized when geraniol or farnesol are added to the medium (Lakin-Thomas and Brody, 2000; Mattern et al., 1982).	The mechanisms and components are unknown.
choline deficiency oscillator (CDO)	Developmental rhythm in *chol-1* or *cel* under choline starvation can be manipulated by unsaturated fatty acid substitution. The rhythm ranges from 33 to more than 120h, depending on the genetic background (Lakin-Thomas, 1996).	The rhythm is not circadian. It can be entrained by light to periods near its FRP, but not within the circadian range. The developmental rhythm is not temperature-compensated, but it is pH-compensated (Lakin-Thomas, 1998; Ruoff and Slewa, 2002). It may present a metabolic oscillator that lies downstream of the FRQ/WCC oscillator which fine-tunes the rhythm (Shi et al., 2007). However, components of the CDO are not known.
nitrate reductase oscillator (NRO)	Nitrate reductase activity (NRA) displays a circadian rhythm that is maintained in strains lacking FRQ or WC-1 in DD or LL when nitrate is used as the sole nitrogen source (Christensen et al., 2004).	The temperature-compensation and entrainment characteristics are unknown, and the outputs from the NRA have not been identified.
rhythms in diacylglycerol (DAG) levels	Rhythms in DAG levels have been observed in Δ*frq* strains. The DAG rhythm is out of phase with the rhythm in conidiation (Ramsdale and Lakin-Thomas, 2000). The rhythm can be influenced by *frq* alleles.	
WC-dependent FLO (WC-FLO)	*Clock- controlled gene-16* (*ccg-16*) mRNA rhythms are produced by a temperature-responsive, temperature-compensated circadian FLO which requires WC-1 and WC-2 (Correa et al., 2003; de Paula, et al. 2006).	The molecular details and the components other than WC-1 and WC-2 of the WC-FLO are unknown.

two mutant strains which displayed developmental rhythm in bright LL (1200 lux) with a circadian period in the absence of a functional FRQ/WCC oscillator. These two mutant strains are named LM-1 (light mutant-1) and LM-2 (Figure 5). Based on these preliminary data, I have tested the hypothesis that the light mutant oscillator (LMO) is a circadian oscillator that can function independently of the FRQ/WCC oscillator. My results presented in the following sections support this hypothesis.

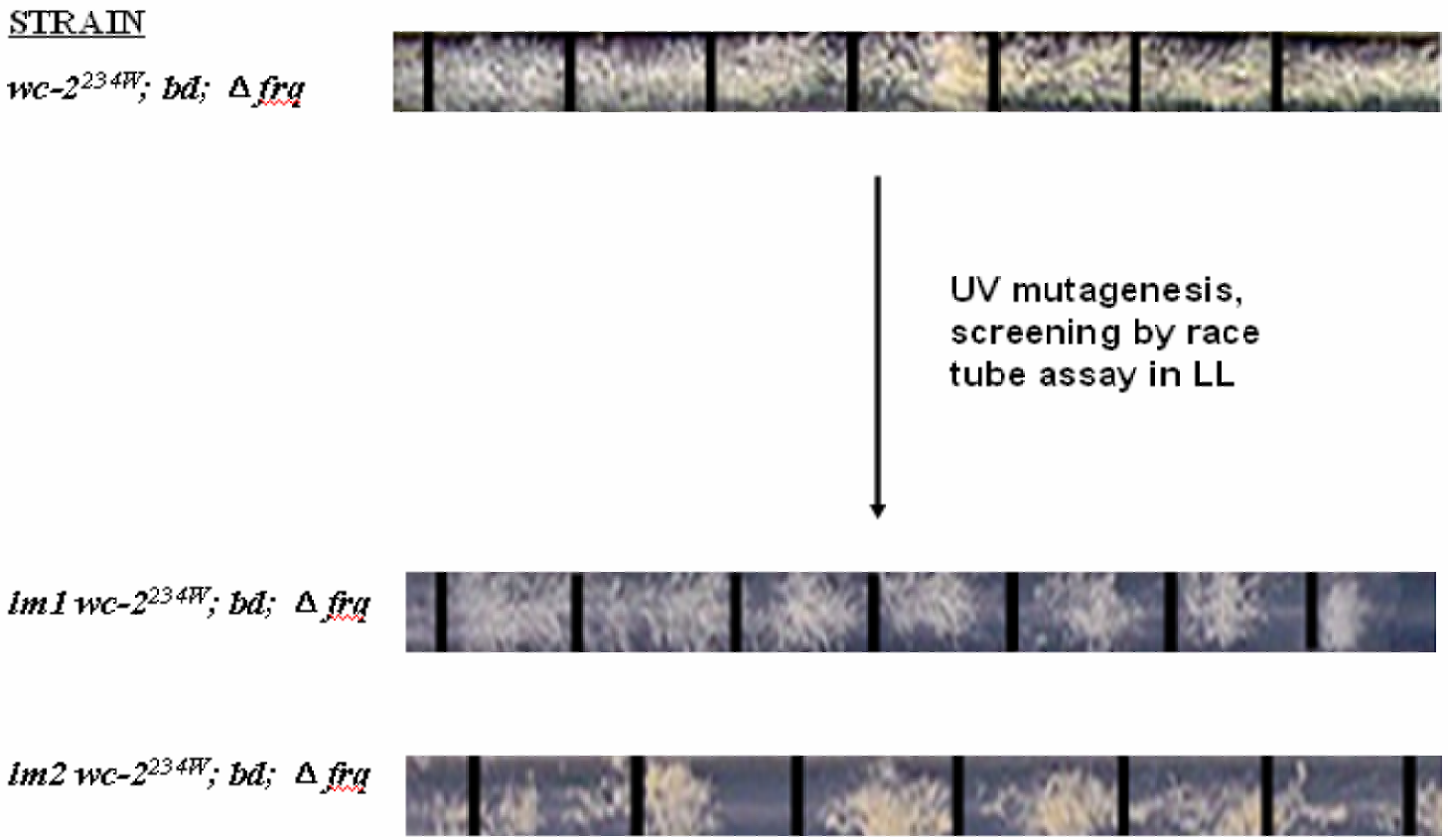


Figure 5. Identification of the LM-1 and LM-2 Mutant Strains Which Display Conidiation Rhythms in LL (1200 lux). Representative photographs of race tubes are shown for the indicated strains grown in LL at 25 " C. The growth direction is from left to right and solid vertical lines correspond to 24 h growth. The differences in the colors of the bands are due to arbitrary differences in photography and do not reflect significant differences in the developmental phenotype.

2. CONDITIONS FOR GROWTH OF THE LM-1 AND LM-2 MUTANT STRAINS THAT OPTIMIZE THE RHYTHM OF DEVELOPMENT IN LL

2.1 Introduction

To begin to identify components of the FLOs, our lab undertook a genetic screen for FRQ suppressors. About 8000 UV mutagenized *wc-2*^{234W}*; bd;* Δ*frq* mutant colonies were screened for developmental rhythms in LL and for loss of rhythmicity in temperature cycles.

Dr. Kyung Seo identified two mutant strains which displayed developmental rhythm in bright LL (1200 lux) with a circadian period in the absence of a functional FRQ/WCC oscillator. These two mutant strains are named LM-1 and LM-2 (Figure 5). While other *N. crassa* mutant strains have also been shown to display conidiation rhythmicity in LL, for instance, *poky, lis-1, lis-2, lis-3*, *rib-1*and *rib-2* (Paietta, 1981 and Paietta, 1983), these strains differ significantly from LM-1 and LM-2, in that they have defective growth and their rhythmicity is restricted to dim light of less than 500 lux (Paietta, 1981 and Paietta, 1983).

However, both the LM-1 and LM-2 mutant strains that were isolated, stored and initially characterized by Dr. Seo were found to be difficult to work with. The rhythmic phenotypes were not always apparent and seemed to depend on media conditions. We suspected that the strains had other mutations, and/or that they were heterokaryons.

Therefore, my first goal was to re-isolate the LM-1 and LM-2 mutant strains following several backcrosses, and to determine the optimal growth conditions for observing developmental rhythms in the LM-1 and LM-2 mutant strains in LL.

2.2 Materials and Methods

Strains

All strains used in this study are listed in Table 2. All strains carry the *band (bd*) mutation (unless indicated otherwise). The mating types of the strains are indicated as *mat A* or *mat a*.

Culture conditions

All vegetative cultures were maintained on Vogel's minimal media (1X Vogel's salts, 2% glucose) and handled according to standard protocols (Vogel, 1956; Davis and Deserres, 1970). Strains containing the bacterial hygromycin resistance gene (*hph*) cassette, such as the strains with the frq^{10} or Δ*wc-1* mutations were maintained on Vogel's minimal media supplemented with 200 μg/ml hygromycin B. Sexual crosses were performed on Westergaard's crossing agar plates containing synthetic crossing media (Westergaard and Mitchell, 1947) supplemented with 0.5% sucrose.

Race tube assay

Race tube assays were performed in environment-controlled chambers (Percival Scientific, Inc., Perry, IA). Light intensity was measured by dual range light meter

Table 2. *N. crassa* Strains Used in this Study

Strains	Genotype	Phenotype	Source/Reference
FGSC[a] 1858	*mat A; bd*	wild-type; 22 h period in DD at 25°C	FGSC 1858
FGSC 1859	*mat a; bd*	wild-type; 22 h period in DD at 25°C	FGSC 1859
DBP[b] 580	*mat A; bd; Δwc-1*	arrhythmic in DD and LL at 25°C	Lee et al., 2003
DBP 590	*lm1 mat A; bd; Δwc-1*	rhythmic in LL	stored by Dr. Seo
DBP 694	*lm1 mat A; bd*	17 h period in LL at 25°C	stored by He Huang
DBP 695	*lm1 mat a; bd*	17 h period in LL at 25°C	stored by He Huang
DBP 696	*lm1 mat A; bd; Δwc-1*	25 h period in LL at 25°C	stored by He Huang
DBP 697	*lm1 mat a; bd; Δwc-1*	25 h period in LL at 25°C	stored by He Huang
DBP 714	*lm2 mat a; bd*	25 h period in LL at 25°C	stored by He Huang
DBP 715	*lm2 mat A; bd*	25 h period in LL at 25°C	stored by He Huang
KS[c] 160	*lm2 mat A; bd*	rhythmic in LL	stored by Dr. Seo

[a] Fungal Genetics Stock Center, University of Missouri, Kansas City, MO. [b] Dr. Deborah Bell-Pedersen lab stock number. [c] Dr. Kyung Seo's stock number.

(VWR Scientific, Inc., Friendswood, TX).

2.3 Results

Remove any superfluous mutations in the LM-1 mutant strain by backcrossing

To re-isolate the LM-1 mutant strain, the mutant strain DBP 590 (*lm1 mat A; bd;* Δ*wc-1*; which was stored by Dr. Kyung Seo and showed rhythmicity in LL at 25 °C), was backcrossed to the WT strain FGSC 1859 (*mat a; bd*), which was arrhythmic in LL (Figure 6). This cross is referred to as cross 526. In this backcross, 1:1 segregation of the LM-1 phenotype was observed. This segregation pattern is consistent with the LM-1 mutation being the only mutation in this strain that affects the developmental rhythm in LL. Progeny from this cross were grown on race tubes in LL at 25 °C in Percival incubators. The growth front was then marked every 24 h. Periods of the rhythms (the time between two consecutive conidial bands, h) and growth rates (the average distance the growth front of the conidia moved within 24 h, cm/day) were calculated. From these experiments, two progeny, which showed the most robust and stable rhythmicity in LL at 25 °C, 526.36 (*lm1 mat A; bd*) and 526.25 (*lm1 mat a; bd;* Δ*wc-1*), were chosen for future analysis. Theses two strains were crossed to each other, referred to as cross 538 (Figure 6). All of the progeny of cross 538 exhibited the same LM-1 phenotypes either as 526.36 (*lm1 mat A; bd*) or 526.25 (*lm1 mat a; bd;* Δ*wc-1*). Four progeny from the cross of 538, 538.19 (*lm1 mat A; bd;* Δ*wc-1*), 538.21 (*lm1 mat a; bd;* Δ*wc-1*), 538.22 (*lm1 mat a; bd*) and 538.27 (*lm1 mat A; bd*), which exhibited the most robust and stable rhythmicity in LL at 25 °C were stored as strains of DBP 696, DBP 697, DBP 695 and

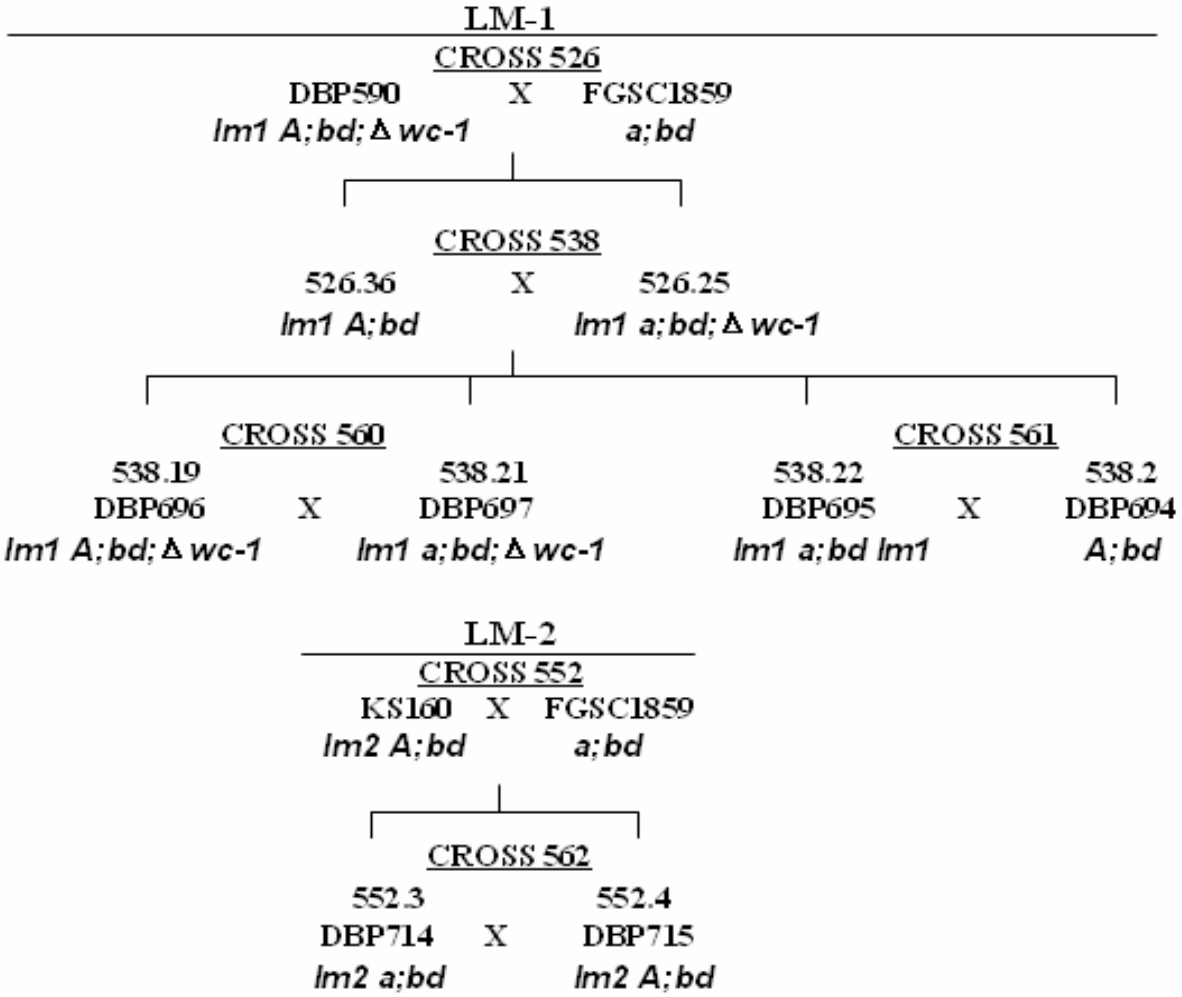


Figure 6. Flow Chart for Re-isolation of the LM-1 and LM-2 Mutant Strains. X represents crossing.

DBP 694, respectively (Figures 6 and 7).

Phenotypes of these four stored strains were confirmed in independent blind tests by other lab colleagues. In addition, DBP 696 (*lm1 mat A; bd;* Δ*wc-1*) was crossed to DBP 697 (*lm1 mat a; bd;* Δ*wc-1*) referred as cross 560 (Figure 6). DBP 695 (*lm1 mat a; bd*) was crossed to DBP 694 (*lm1 mat A; bd*) referred as cross 561. Ten progeny from these crosses were phenotypically tested, and all of them exhibited the same phenotypes as their parents and siblings, which indicates that any superfluous mutations in the LM-1 mutant strain were removed.

Remove any superfluous mutations in the LM-2 mutant strain by backcrossing

To re-isolate the LM-2 mutant strain, the mutant strain KS 160 (*lm2 mat A; bd*), which was stored by Dr. Seo and showed rhythmicity in LL at 25 °C, was backcrossed to the WT strain of FGSC 1859 (*mat a; bd*) (Figure 6). This cross is referred to as cross 552. In 20 progeny from the cross, 1:1 segregation of the LM-2 phenotype was observed, indicating that the LM-2 mutation in KS 160 is the only mutation in the strain that affects the period in LL. Progeny from the crosses were grown on race tubes in LL at 25 °C in Percival incubators. Periods of the rhythms and growth rates were calculated. Two progeny from the cross, 552.3 (*lm2 mat a; bd*) and 552.4 (*lm2 mat A; bd*), which exhibited the most robust and stable rhythmicity in LL at 25 °C, were stored as strains of DBP 714 and DBP 715 (Figures 6 and 7).

LL 25 ° C

STRAIN	PERIOD (h)
DBP1859(*a; bd*)	AR
DBP1858(*A; bd*)	AR
DBP695(*lm1 a; bd*)	17.1 ± 1.3 (n=37)
DBP694(*lm1 A; bd*)	17.1 ± 1.3 (n=194)
DBP696(*lm1 A; bd; Δwc-1*)	25.1 ± 0.8 (n=191)
DBP697(*lm1 a; bd; Δwc-1*)	25.1 ± 0.8 (n=35)
DBP714(*lm2 a; bd*)	24.5 ± 0.8 (n=164)
DBP715(*lm2 A; bd*)	24.5 ± 0.8 (n=32)

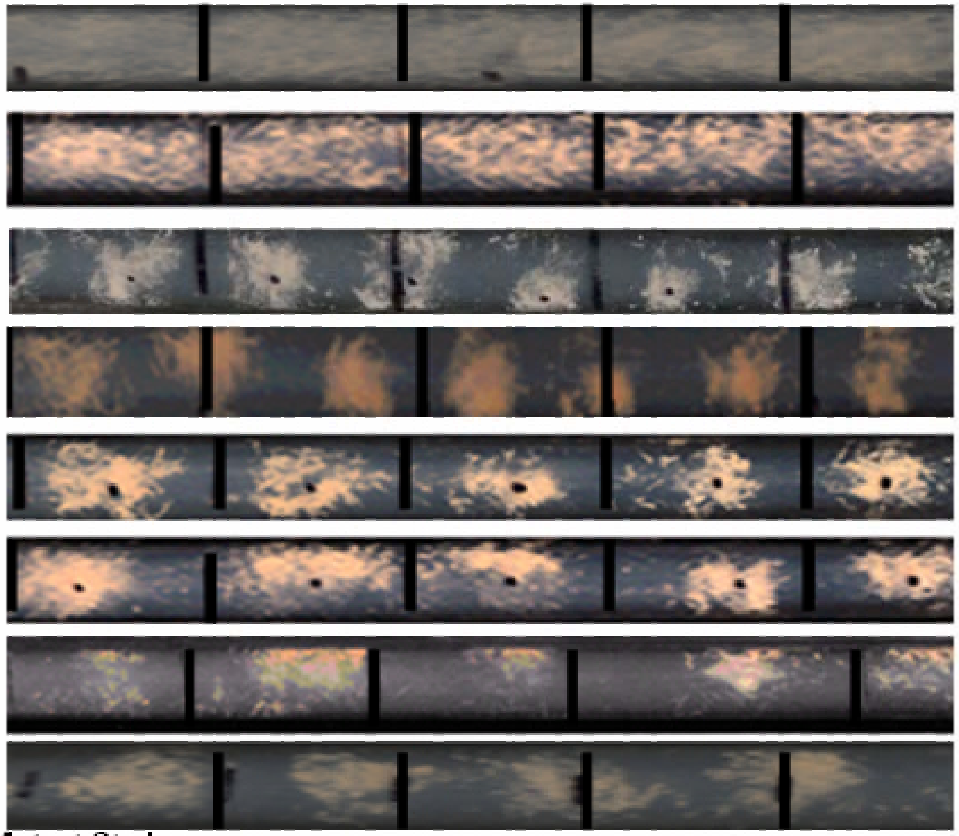


Figure 7. Re-isolation of the LM-1 and LM-2 Mutant Strains.
Representative photographs of race tubes are shown for the indicated strains grown in LL at 25 ° C. The growth direction is from left to right and solid vertical lines correspond to 24 h growth. The period lengths of the rhythmic strains are indicated with standard deviations. AR represents arrhythmic phenotype; n represents the sample size. The differences in the colors of the bands are due to arbitrary differences in photography and do not reflect significant changes in the developmental phenotypes of the mutants.

Phenotypes of these two stored strains were confirmed in independent tests by other lab colleagues. In addition, DBP 714 (*lm2 mat a; bd*) was crossed to DBP 715 (*lm2 mat A; bd*) referred to as cross 562 (Figure 6). Ten progeny from this cross were tested on race tubes, and all of them exhibited the same phenotypes as their parents and siblings, which suggests that any superfluous mutations in the LM-2 mutant strain were removed.

However, the LM-2 mutant strain was still found to be unstable. In subsequent crosses by Dr. Xiaoguang Liu in our lab, he observed that the phenotype of the LM-2 mutant strain is very dependent on moisture levels in race tube and temperature. Furthermore, the period is dependent on the presence or absence of WC-1. Due to these complexities, I have focused on charactering the LM-1 mutant strain and Dr. Liu is focusing on the LM-2 mutant strain.

Determine the optimal growth conditions for observing developmental rhythms in the LM-1 mutant strain in LL

The newly isolated *lm1 bd* strains, DBP 694 and DBP 695, were tested on race tubes to determine the optimal growth conditions for revealing the highest amplitude and most consistent rhythm of development. Based on past experience, I changed the chemical composition of the race tube medium (Sargent, 1966) singly and in combination: Vogel's salts (1X or 2X), glucose (0.1% or 0.3%), arginine (0.17% or 0.5%) and agar (1.5% or 2%). I also altered the volume of media in the race tube (11ml or 13ml), the moisture level of the race tubes when they were being inoculated (keeping them on

bench for 1 week after autoclaving or 4 weeks after autoclaving), the age of the LM-1 mutant strain (1 week old or 4 weeks old), the light intensity these strains received in Percival incubators (500 lux or 1500 lux), and the temperature in the Percival incubators (15 °C, 19 °C, 25 °C or 30 °C). The optimal conditions for observing developmental rhythms in the LM-1 mutant strain in LL were found to be the following: (1) 1 week old race tube medium containing 1X Vogel's salts, 0.1% glucose, 0.17% arginine and 1.5% agar, (2) 11ml medium in the race tube, (3) inoculation with a one week old culture of the LM-1 mutant strain, (4) incubation in Percival incubators at 25 °C with the light intensity of 1500 lux (data not shown).

2.4 Discussion

Remove any superfluous mutations in the LM-1 and LM-2 mutant strains by backcrossing

In all the backcrosses performed in this process, 1:1 segregation of the LM-1 or LM-2 phenotype with the rhythmic progeny showing equivalent period length was always observed. All of the progeny exhibited the same phenotypes with their parents and siblings for the crosses of the same genotypes with the exception of mating type; this result suggested that any superfluous mutations in both the LM-1 and LM-2 mutant strains were removed. The LM-1 and LM-2 mutations were identified in a ΔFRQ strain. The backcrosses were also used to obtain the LM-1 and LM-2 mutations in a FRQ^{+} strain. Importantly, the rhythm persisted in LL independent of whether or not FRQ was present in the strain.

Determine the optimal growth conditions for observing developmental rhythms in the LM-1 mutant strain in LL

For changes in the chemical composition of the race tube medium with respect to conidiation rhythms, Vogel's salts was better than 2X Vogel's salts, 0.1% glucose was better than 0.3% glucose and 0.17% arginine was better than 0.5% arginine. Since the standard chemical composition of the race tube media is 1X Vogel's salts, 0.3% glucose and 0.5% arginine (Sargent, 1966), these data indicated that the LM-1 mutant strain shows a strong rhythm when grow at a lower concentration of sugar (glucose) and nitrogen (arginine). WT strains were arrhythmic in these conditions. The amount of agar in the race tubes, the volume of media in the race tube and the time interval between autoclaving and inoculating race tubes collectively influenced the moisture level of the race tubes when they were inoculated. The age of the LM-1 mutant strain also affected the observed developmental rhythms significantly: a one week old strain performed much better than a 4 week old strain. The most critical growth condition for observing developmental rhythms for the LM-1 mutant strain was the light intensity it received in Percival incubators. Strong light of 1500 lux was produced strong rhythms than did dim light of 500 lux conditions in other *N. crassa* mutant strains have been shown to display conidiation rhythms in 500 lux LL (Paietta, 1981; Paietta, 1983). Moreover, the LM-1 mutant strain was identified in bright LL (1200 lux). The temperature in the Percival incubators had the least effect on the observed LM-1 developmental rhythms. The growth conditions found to reveal the most robust rhythm for the LM-1 mutant strain were used for all subsequent experiments.

3. THE LMO IS A CIRCADIAN OSCILLATOR

3.1 Introduction

Circadian rhythms are defined by three main characteristics as described in Section 1 (reviewed by Dunlap *et al.*, 2004). Experiments were conducted to examine if the LMO uncovered by the LM-1 and LM-2 mutations meet the following criteria of a true circadian oscillator.

(1) The developmental rhythms regulated by the LMO should persist with an intrinsic FRP of roughly 24 h, when the organism is kept under constant conditions, such as LL at 25 °C or DD at 25 °C. If the FRP is not close to 24 h, it should be entrained by 24 h environmental cycles.

(2) The developmental rhythms regulated by the LMO should be entrained by external cues, including temperature or light, in a time-dependent fashion.

(3) The developmental rhythms regulated by the LMO should be temperature-compensated, meaning the FRP is nearly constant across an extensive range of physiologically relevant temperatures.

3.2 Materials and Methods

Strains, culture conditions and race tube assay

All strains used in this study are listed in Table 3. All assays are the same as described in Section 2.

PCR conditions to verify strains containing Δfrq

PCR reaction mixture contained: 1 µl genomic DNA (50 ng/µl), 1 µl each primer (10 µM), 8 µl dNTPs (2.5 mM) (Fisher Scientific, Houston, TX), 0.5 µl TaKaRa ExTaq polymerase (5 U/ µl , Fisher Scientific, Houston, TX) and 5 µl 10 X ExTaq buffer (Fisher Scientific, Houston, TX). These were assembled in a 50 µl total volume on ice. Thermal cycler programs were started and paused when the block temperature reached 95 °C before PCR tubes were inserted. Samples were initially denatured for 3 minutes at 95 °C, then treated with 30 cycles of 30 seconds denature at 95 °C, 30 seconds annealing at 60 °C, 2 minute extension at 72 °C, followed by 5 minutes final extension at 72 °C. The tubes were then stored at 4 °C. The null Δ*frq* mutant strain was produced by gene replacement with the bacterial hygromycin resistance gene (*hph*) (Aronson et al., 1994a). The forward primer of 5'GAAGCATACTATCGCCAGAC3' anneals to the 5' region of *frq* locus, and the reverse primer of 5'AGCACTCGTCCGAGGGCAAA3' anneals to the *hph* gene insertion. The forward primer of 5'ATGACAAAAACA ACGCCATACA3' and the reverse primer of 5'TTATTCCCAAGCAGACCCCA3' to the *invertase* gene were used as a positive control for the PCR.

Table 3. *N. crassa* Strains Used in this Study

Strains	Genotype	Phenotype	Source/Reference
FGSC[a] 1858	*mat A; bd*	wild-type; 22 h period in DD at 25°C	FGSC 1858
FGSC 1859	*mat a; bd*	wild-type; 22 h period in DD at 25°C	FGSC 1859
DBP[b] 287	*mat a; bd; Δfrq*	arrhythmic in DD and LL at 25°C	Aronson et al., 1994a
DBP 294	*mat a; bd; frq^7*	29 h period in DD at 25°C	Feldman and Hoyle, 1973
DBP 339	*mat A; bd; frq^7*	29 h period in DD at 25°C	Feldman and Hoyle, 1973
DBP 580	*mat A; bd; Δwc-1*	arrhythmic in DD and LL at 25°C	Lee et al., 2003
DBP 694	*lm1 mat A; bd*	17 h period in LL at 25°C	stored by He Huang
DBP 696	*lm1 mat A; bd; Δwc-1*	25 h period in LL at 25°C	stored by He Huang
DBP 697	*lm1 mat a; bd; Δwc-1*	25 h period in LL at 25°C	stored by He Huang
DBP 831	*lm1 mat A; bd; Δfrq*	25 h period in LL at 25°C	stored by He Huang
DBP 832	*lm1 mat a; bd; Δfrq*	25 h period in LL at 25°C	stored by He Huang
DBP 833	*lm1 mat A; bd; frq^7*	14 h period in LL at 25°C	stored by He Huang
DBP 834	*lm1 mat a; bd; frq^7*	14 h period in LL at 25°C	stored by He Huang

[a] Fungal Genetics Stock Center, University of Missouri, Kansas City, MO. [b] Dr. Deborah Bell-Pedersen lab stock number.

3.3 Results

*Cross the LM-1 mutant strain to FRQ/WCC oscillator mutant strains (*Δwc-1, Δfrq *and* frq^{7}*) and isolate the double mutants*

To determine if the LMO can function to control rhythms independent of the FRQ/WCC oscillator, Δ*wc-1,* Δ*frq* and *frq*^{7}were reintroduced into the LM-1 mutant strain.

The Δ*wc-1* mutant strain is arrhythmic in DD and LL and lacks all known light responses, including light entrainment of the circadian clock (Crosthwaite et al., 1995 and 1997). WC-1 is the main blue-light photoreceptor in *N. crassa* that mediates light input to FRQ/WCC oscillator (He et al., 2002, Froehlich et al., 2002 and Lee et al., 2003). DBP 696 (*lm1 mat A; bd;* Δ*wc-1*) and DBP 697 (*lm1 mat a; bd;* Δ*wc-1*) were described in section 2.3. The *wc-1* deletion is currently being verified by PCR.

To get Δ*frq* into the LM-1 mutant background, DBP 694 (*lm1 mat A; bd*) was crossed to DBP 287 (*mat a; bd;* Δ*frq*) referred to as cross 564. Two progeny 564.42 (*lm1 mat A; bd;* Δ*frq*) and 564.40 (*lm1 mat a; bd;* Δ*frq*) showed robust rhythmicity in LL which indicated that they contained the LM-1 mutant. The Δ*frq* strain conidiates arrhythmically under standard growth conditions in DD and LL (Aronson et al., 1994a). Both of the progeny 564.42 and 564.40 exhibited growth on media containing hygromycin indicating that they contained Δ*frq*. The *frq* deletion in these strains was also verified by PCR (Figure 8). 2 kb expected bands were obtained from all the strains tested using *invertase* primers as a positive control, indicating that PCR conditions were working.

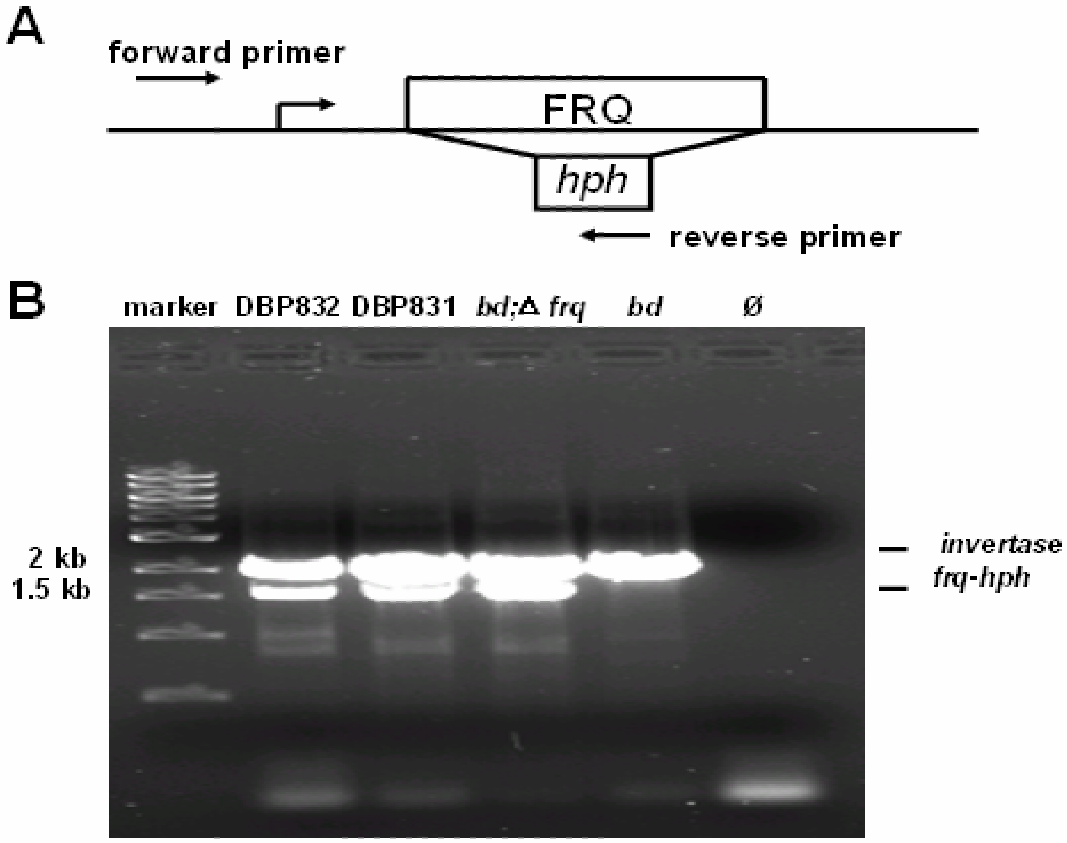


Figure 8. Verification of *lm1 bd;* Δ *frq* Double Mutant Strains (DBP 831 and DBP 832) by PCR.
(A) Cartoon diagram of the Δ *frq* strain. ↱ represents the transcriptional start site for the *frq* mRNA. The box labeled "FRQ" represents the complete FRQ ORF. The box labeled "*hph*" represents the replacement site of the *hph* coding region for *frq*. The size of the PCR product for Δ *frq* strains is predicted to be around 1.5 kb.
(B) Agarose gel showing that *lm1 bd;* Δ *frq* double mutant strains (DBP 831 and DBP 832) contain *hph* in place of the *frq* ORF. Ø represents no DNA. *Invertase* serves as a positive control for the PCR. The size of the PCR product for *Invertase* is predicted to be around 2 kb.

Bands of the expected 1.5 kb size were obtained from the strains containing Δ*frq*, indicating that the null Δ*frq* mutant strain was produced by gene replacement *hph*. Thus, 564.42 (*lm1 mat A; bd;* Δ*frq*) and 564.40 (*lm1 mat a; bd;* Δ*frq*) were stored as DBP 831 and DBP 832.

The frq^7 mutation is a G to A point mutation, and results in a long period of 29 h in DD, but is arrhythmic in LL (reviewed by Loros and Dunlap, 2001).To obtain frq^7 in the LM-1 genetic background, DBP 694 (*lm1 mat A; bd*) was crossed to DBP 294 (*mat a; bd;* frq^7) referred as cross 566. Two progeny 566.33 (*lm1 mat A; bd;* frq^7) and 566.37 (*lm1 mat a; bd;* frq^7) showed robust and consistent rhythmicity in LL which indicated that they contained the LM-1 mutant. Both of the progeny 566.33 and 566.37 exhibited 31.1 h rhythms in DD, whereas *lm1 mat a; bd* strains displayed a 22.8 h rhythm in DD (Figure 9). These data indicate that 566.33 and 566.37 contain frq^7. Thus, 566.33 (*lm1 mat A; bd;* frq^7) and 566.37 (*lm1 mat a; bd;* frq^7) were stored as DBP 833 and DBP 834. For further proof that these strains contain the frq^7 allele, I will sequence the *frq* alleles in these strains.

Assay the developmental rhythm of the mutant strains in LL

The LM-1 strains, and control strains were examined (Figure 10) by race tube assay under the standard growth conditions defined in section 2.3. in LL at 25 °C. In LL at 25 °C, DBP 580 (*mat A; bd;* Δ*wc-1*), DBP 287 (*mat a; bd;* Δ*frq*), and DBP 339 (*mat A; bd;* frq^7) strains were arrhythmic (Figure 10), while strains carrying the LM-1 mutation in

these genetic backgrounds exhibited circadian rhythmicity. DBP 694 (*lm1 mat A; bd*) had a period of 17.1h ± 1.3 (n=194), DBP 696 (*lm1 mat A; bd;* Δ*wc-1*) had a period of 25.1 h ± 0.8 (n=191), DBP 831 (*lm1 mat A; bd;* Δ*frq*) had a period of 25.0 h ± 2.0 (n=81), and DBP 833 (*lm1 mat A; bd; frq*^{7}) had a period of 13.6 h ± 0.7 (n=172). However, the rhythm of DBP 833 was less robust.

Assay the developmental rhythm in the mutant strains in DD

FGSC 1858 (*mat A; bd*), DBP 694 (*lm1 mat A; bd*), DBP 580 (*mat A; bd;* Δ*wc-1*), DBP 696 (*lm1 mat A; bd;* Δ*wc-1*), DBP 287 (*mat a; bd;* Δ*frq*), DBP 831 (*lm1 mat A; bd;* Δ*frq*), DBP 339 (*mat A; bd; frq*^{7}) and DBP 833 (*lm1 mat A; bd; frq*^{7}) were tested by race tube assay under the standard growth conditions defined in Section 2 in DD at 25 °C, conditions in which the FRQ/WCC oscillator is active in WT strains. Periods and growth rates of these strains were calculated. In DD at 25 °C, FGSC 1858 (*mat A; bd*) WT strain showed a period of 22.0 h ± 0.2 (n=70) (Figure 10), DBP 694 (*lm1 mat A; bd*) showed a period of 22.8 h ± 0.3 (n=91). DBP 580 (*mat A; bd;* Δ*wc-1*) and DBP 287 (*mat a; bd;* Δ*frq*) strains were arrhythmic (Figure 10) in DD, while strains containing the LM-1 mutation in these genetic backgrounds exhibited circadian rhythmicity: DBP 696 (*lm1 mat A; bd;* Δ*wc-1*) had a period of 21.9 h ± 1.3 (n=41), DBP 831 (*lm1 mat A; bd;* Δ*frq*) had a similar period of 21.7 h ± 1.7 (n=34). DBP 339 (*mat A; bd; frq*^{7}) exhibited a period of 28.8 h ± 0.1 (n=26), while DBP 833 (*lm1 mat A; bd; frq*^{7}) exhibted a period of 31.1 h ± 0.2 (n=35).

STRAIN	PERIOD (h)	
DBP1858(*A; bd*)	22.0±0.2 (n=70)	
DBP694(*im1 A; bd*)	22.8±0.3 (n=91)	
DBP580(*A; bd; Δwc-1*)	AR	
DBP696(*im1 A; bd; Δwc-1*)	21.9±1.3 (n=41)	
DBP287(*a; bd; Δfrq*)	AR	
DBP831(*im1 A; bd; Δfrq*)	21.7±1.7 (n=34)	
DBP339(*A; bd; frq^7*)	28.8±0.1 (n=26)	
DBP833(*im1 A; bd; frq^7*)	31.1±0.2 (n=35)	

Figure 9. The LM-1 Mutant in WT, Δ *wc-1*, Δ *frq* and *frq*^{7} Genetic Backgrounds in DD at 25 ° C.
Representative photographs of race tubes are shown for the indicated strains grown in DD at 25 ° C. The figure labeled as described in Figure 7.

STRAIN	PERIOD (h)	
DBP1858(*A; bd*)	AR	
DBP694(*im1 A; bd*)	17.1 ± 1.3 (n=194)	
DBP580(*A; bd; Δ wc-1*)	AR	
DBP696(*im1 A; bd; Δ wc-1*)	25.1 ± 0.8 (n=191)	
DBP287(*a; bd; Δ frq*)	AR	
DBP831(*im1 A; bd; Δ frq*)	25.0 ± 2.0 (n=81)	
DBP339(*A; bd; frq^7*)	AR	
DBP833(*im1 A; bd; frq^7*)	13.6 ± 0.7 (n=172)	

Figure 10. The LM-1 Mutant in WT, Δ *wc-1*, Δ *frq* and *frq^7* Genetic Backgrounds in LL at 25 ° C.
Representative photographs of race tubes are shown for the indicated strains grown in LL at 25 ° C. The figure is labeled as described in Figure 7.

The LM-1 mutant strains maintain a circadian FRP in constant conditions

While still rhythmic, the amplitude of the LM-1 rhythm in strains that lack a functional FRQ/WCC oscillator was not as robust as FRQ^{+} or $WC\text{-}1^{+}$ strains. Our results are consistent with previous studies showing that *bd; Δfrq* and *bd; Δwc-1* strains are arrhythmic under these conditions (Aronson et al., 1994a and Lee et al., 2003). Together, these data demonstrate that the LM-1 mutant strain oscillator persists in DD and LL, independent of a functional FRQ/WCC oscillator.

The developmental rhythm of the LM-1 mutant strains can be entrained by LD cycles

To determine if the developmental rhythm regulated by the LMO can be entrained, the LM-1 mutant strains were placed in various LD cycles. The strains were first grown on race tubes in LL at 25 °C for 24 h, after which, the growth front was marked and the culture was transferred to different LD cycles in Percival incubators. The growth front was then marked every time the lights were turned off. FGSC 1858 (*mat A; bd*), DBP 694 (*lm1 mat A; bd*), DBP 580 (*mat A; bd; Δwc-1*), DBP 696 (*lm1 mat A; bd; Δwc-1*), DBP 287 (*mat a; bd; Δfrq*), DBP 831 (*lm1 mat A; bd; Δfrq*) were placed in light (1500 lux, 12 h) : dark (12 h) cycles at 25 °C (LD12, Figure 11) and light (1500 lux, 14 h) : dark (14 h) cycles at 25 °C (LD14, Figure 12).

Similar to previous results (Aronson et al., 1994a and Lee et al., 2003), DBP 580 (*mat A; bd; Δwc-1*) and DBP 287 (*mat a; bd; Δfrq*) did not show developmental rhythms in LD12 at 25 °C (Figure 11) or LD14 at 25 °C (Figure 12). However, the LM-1 mutant

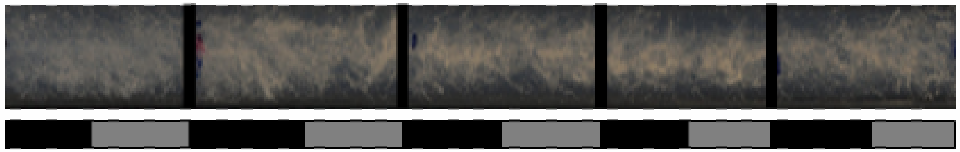
STRAIN	PERIOD (h)	
DBP1858(*A; bd*)	24.3 ± 0.3 (n=55)	
DBP694(*im1 A; bd*)	24.1 ± 0.2 (n=102)	
DBP580(*A; bd; Δ wc-1*)	AR	
DBP696(*im1 A; bd; Δ wc-1*)	24.2 ± 0.4 (n=74)	
DBP287(*a; bd; Δ frq*)	AR	
DBP831(*im1 A; bd; Δ frq*)	24.9 ± 2.5 (n=43)	

Figure 11. The LM-1 Mutant Strain Rhythm is Entrained by 12:12 LD Cycles at 25 " C.
Representative photographs of race tubes are shown for the indicated strains grown in 12:12 LD cycles at 25 " C. The grey bar represents time in LL and the black bar represents time in DD. The figure is labeled as described in Figure 7.

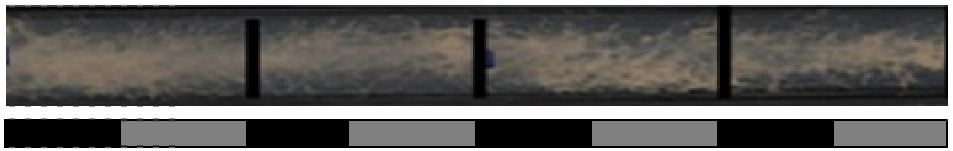
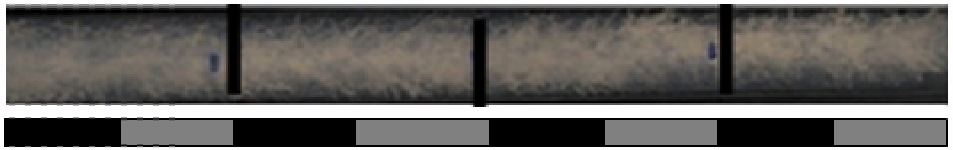
STRAIN	PERIOD (h)	
DBP1858(*A; bd*)	28.0 ± 0.1 (n=31)	
DBP694(*im1 A; bd*)	28.1 ± 0.1 (n=43)	
DBP580(*A; bd; Δ wc-1*)	AR	
DBP696(*im1 A; bd; Δ wc-1*)	28.3 ± 0.2 (n=38)	
DBP287(*a; bd; Δ frq*)	AR	
DBP831(*im1 A; bd; Δ frq*)	28.4 ± 0.6 (n=28)	

Figure 12. The LM-1 Mutant Strain Rhythm is Entrained by 14:14 LD Cycles at 25 ° C.
Representative photographs of race tubes are shown for the indicated strains grown in 14:14 LD cycles at 25 ° C. The grey bar represents time in LL and the black bar represents time in DD. The figure is labeled as described in Figure 7.

strain in WT, Δ*wc-1* and Δ*frq* backgrounds displayed developmental rhythms with periods of that equaled the LD cycle. These results demonstrate that the LMO can be entrained by LD cycles, independent of a functional FRQ/WCC oscillator.

The developmental rhythm of the LM-1 mutants can be entrained by temperature cycles

To determine if the developmental rhythm regulated by the LMO can be entrained by temperature cycles, the LM-1 mutant strains were placed in temperature cycles. The strains were first grown on race tubes in LL at 25 °C for 24 h, after which, the growth front was marked and the culture was transferred to 22 °C (12 h) : 27 °C (12 h) cycles in Percival incubator. The growth front was then marked every time the temperature decreased. FGSC 1858 (*mat A; bd*), DBP 694 (*lm1 mat A; bd*), DBP 580 (*mat A; bd;* Δ*wc-1*), DBP 696 (*lm1 mat A; bd;* Δ*wc-1*), DBP 287 (*mat a; bd;* Δ*frq*), DBP 831 (*lm1 mat A; bd;* Δ*frq*) were placed in 22 °C (12 h) : 27 °C (12 h) cycles in both LL (Figure 13) and DD (Figure 14).

Different from the effects on LD cycles and consistent with previous results (Crosthwaite et al., 1995; Merrow et al., 1999), DBP 580 (*mat A; bd;* Δ*wc-1*) and DBP 287 (*mat a; bd;* Δ*frq*) have a 24 h developmental rhythm in temperature cycles. These indicate that the circadian clock in *N. crassa* in the condition of a nonfunctional FRQ/WCC oscillator can be entrained by temperature cycles, but not LD cycles. The LM-1 mutant strain in WT, Δ*wc-1* and Δ*frq* backgrounds also exhibited periods of 24 h

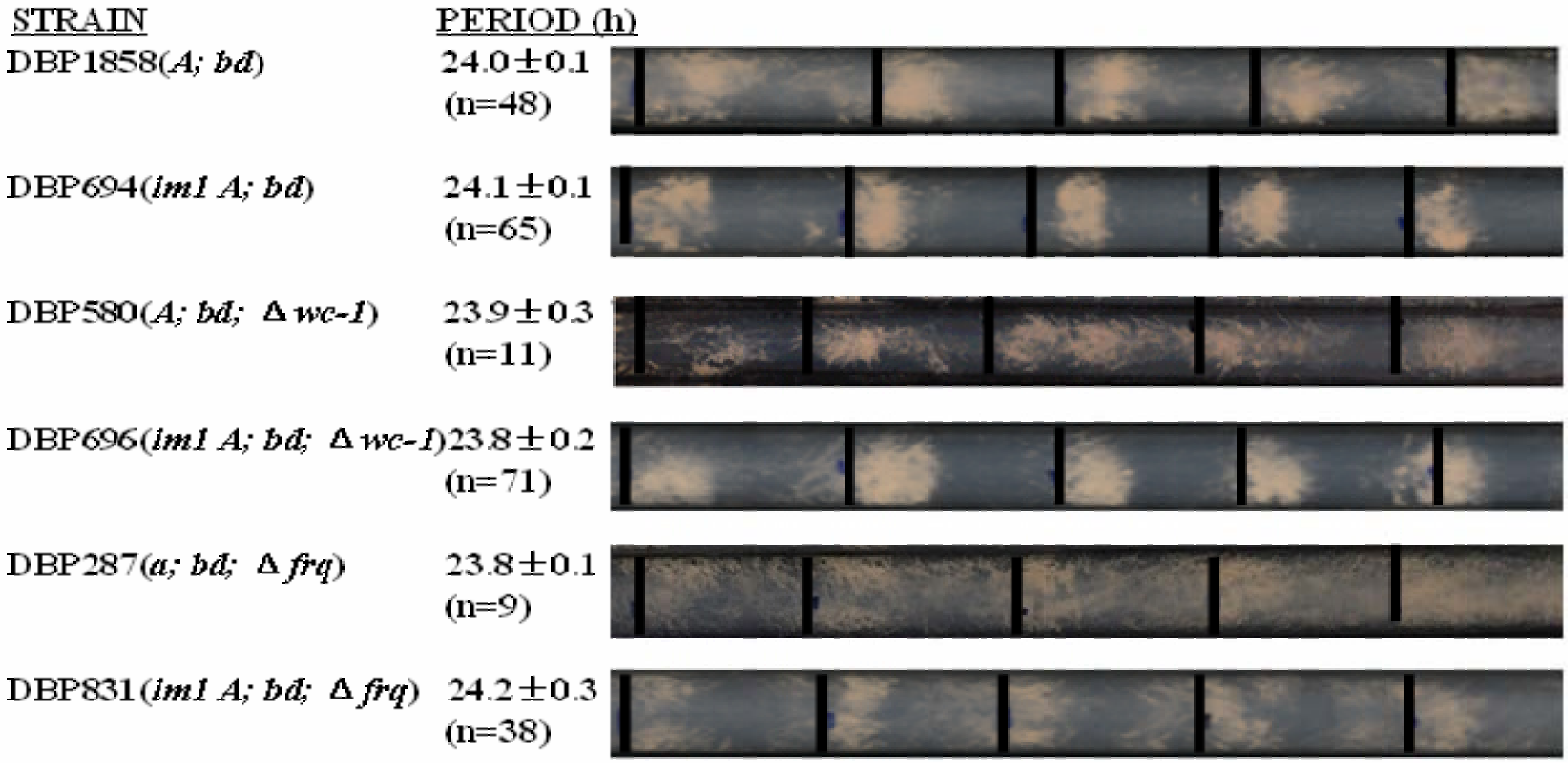


Figure 13. The LM-1 Mutant Strain Rhythm is Entrained by 22° C(12h):27° C(12h) Cycles in LL.
Representative photographs of race tubes are shown for the indicated strains grown in 22° C(12h):27° C(12h) cycles in LL. Marks at the beginning of cold. The figure is labeled as described in Figure 7.

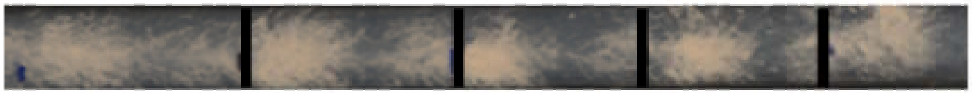
STRAIN	PERIOD (h)	
DBP1858(*A; bd*)	23.8±0.2 (n=51)	
DBP694(*lm1 A; bd*)	24.1±0.1 (n=101)	
DBP580(*A; bd; Δwc-1*)	23.9±0.1 (n=12)	
DBP696(*lm1 A; bd; Δwc-1*)	23.7±0.3 (n=84)	
DBP287(*a; bd; Δfrq*)	24.1±0.4 (n=4)	
DBP831(*lm1 A; bd; Δfrq*)	22.2±0.3 (n=43)	

Figure 14. The LM-1 Mutant Strain Rhythm is Entrained by 22° C(12h):27° C(12h) Cycles in DD.
Representative photographs of race tubes are shown for the indicated strains grown in 22° C(12h):27° C(12h) cycles in DD. Marks at the beginning of cold. The figure is labeled as described in Figure 7.

in these cycles in both LL and DD conditions. All of these strains conidiate in the cold (22 °C) portion of the cycle in LL and DD.

The same strains were placed in 16 °C (12 h) : 32 °C (12 h) cycles in both LL and DD (data not shown). The LM-1 mutant strain in WT, Δ*wc-1* and Δ*frq* backgrounds also exhibited periods of around 24 h and conidiated in the cold (16 °C) partion of the cycle.

The developmental rhythm of the LM-1 mutants is temperature-compensated

To determine if the LMO drives rhythms that are unaffected by temperature in the physiological range, DBP 694 (*lm1 mat A; bd*), DBP 580 (*mat A; bd;* Δ*wc-1*), DBP 696 (*lm1 mat A; bd;* Δ*wc-1*), DBP 287 (*mat a; bd;* Δ*frq*), DBP 831 (*lm1 mat A; bd;* Δ*frq*) were examined at 15 °C, 17 °C, 19 °C, 21 °C, 23 °C, 25 °C and 27 °C for developmental rhythms in LL to determine if the developmental rhythm is temperature-compensated (Figure 15).

The Q_{10} for DBP 694 (*lm1 mat A; bd*) is 1.04, for DBP 696 (*lm1 mat A; bd;* Δ*wc-1*) is 0.93, and for DBP 831 (*lm1 mat A; bd;* Δ*frq*) is 1.00. The Q_{10} values for the developmental rhythm in LL of these mutant strains were close to 1 indicating that the rhythms were essentially temperature- independent.

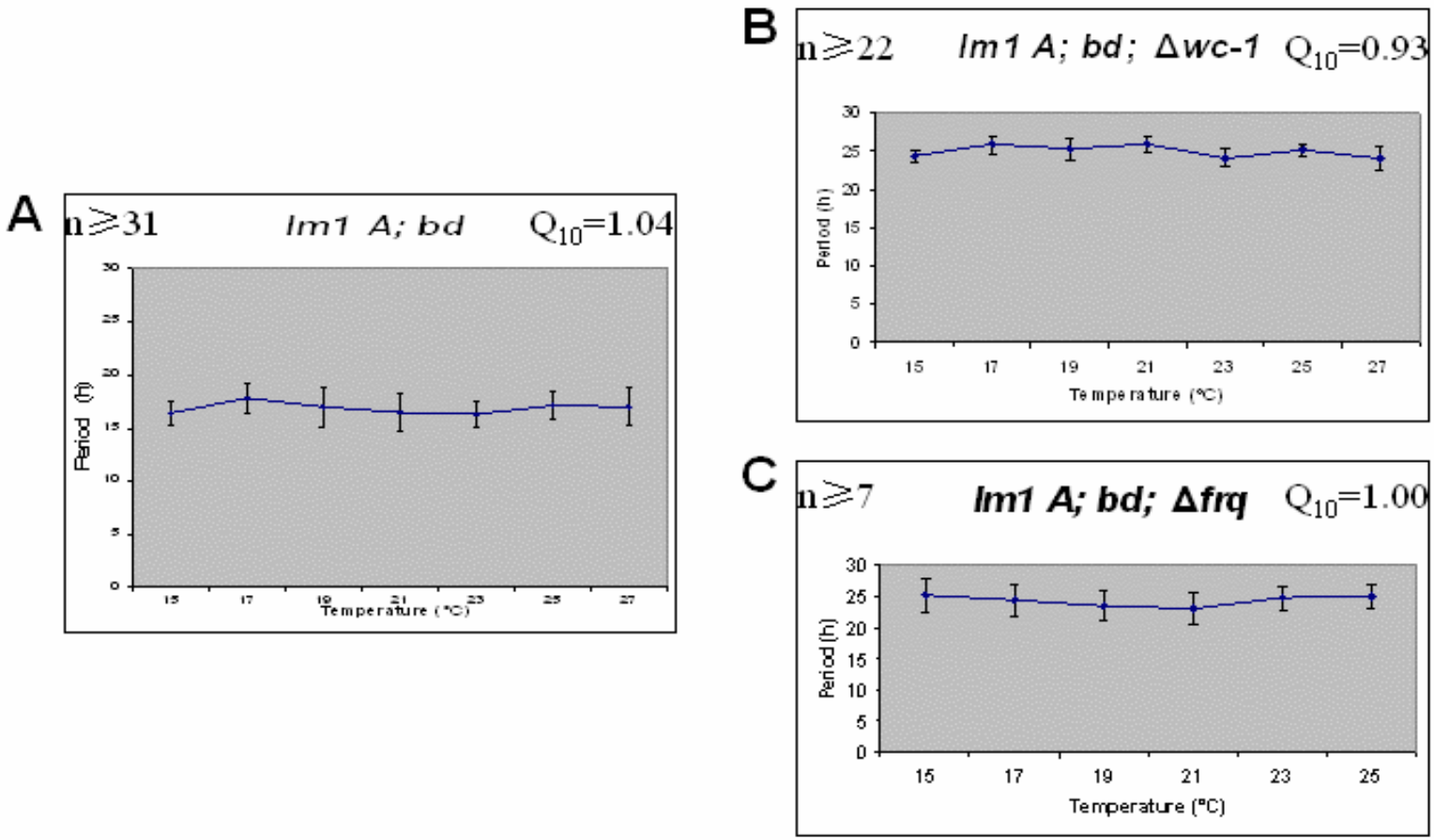


Figure 15. (A-C) The Developmental Rhythm of the LM-1 Mutant Strains is Temperature-Compensated in LL.
The temperatures are indicated on the X-axis and the periods of each strain with standard deviations are indicated on the Y-axis. n represents the sample size.

3.4 Discussion

The LMO is a circadian oscillator

One of the hypotheses tested in this section is that the LMO uncovered by the LM-1 mutation is a circadian oscillator. To support this hypothesis, the three main characteristics that define circadian oscillators, including an intrinsic FRP of roughly 24 h when the organism is kept under constant conditions, entrainment by light and temperature, and temperature-compensation were examined (reviewed by Dunlap *et al.*, 2004).

The developmental rhythms observed in the LM-1 mutant strains persist with an intrinsic FRP of roughly 24 h when the organism is kept under constant conditions. In addition, the LM-1 mutant strains are entrained by different LD cycles, are within the circadian range (LD12) and the other within a longer 28 h period (LD14). I am currently checking if the developmental rhythms regulated by the LMO can be entrained by one LD12 and/or DL12 cycles and then be released in DD and LL (bright light of 1500 lux and dim light of 500 lux). The LM-1 mutant strains are also entrained by different temperature cycles in LL and DD conditions. I am also testing if the developmental rhythms regulated by the LMO can be entrained by one 22 °C (12 h) : 27 °C (12 h) and/or 27 °C (12 h) : 22 °C (12 h) cycles and then be released in DD and LL. These results indicate that the rhythms observed in the LM-1 mutant strains can be entrained by external cues in a time-dependent fashion. Lastly, the Q_{10} of the LM-1 mutant strain was close to 1, indicating that the rhythms were essentially temperature- independent. By assaying the

three key characteristics of circadian rhythms, the LMO, was determined to be a circadian oscillator that can function independent of the FRQ/WCC oscillator to regulate circadian rhythms in development.

There are now extensive lines of physiological evidence for the existence of FLOs in *N. crassa* (Table 1). However, the three properties that define a circadian oscillator are not met or not determined for most of these FLOs. For example, the CDO, rhythm is not circadian. The period of the CDO rhythm ranges from 33 to more than 120 h, depending on the genetic background (Lakin-Thomas, 1996). The CDO rhythm can be entrained by light to periods near its FRP, but not within the circadian range. The developmental rhythm is not temperature-compensated, but it is pH-compensated (Lakin-Thomas, 1998; Ruoff and Slewa, 2002). For the NRO, the temperature-compensation and entrainment characteristics are unknown (Christensen et al., 2004).

A separate light input pathway to the LMO exists

The LM-1 mutation strain can restore light entrainment to a Δ*frq* or a Δ*wc-1* strain (Figure 11 and 12). Because *wc-1* is the blue light receptor required for resetting of the FRQ/WCC oscillator by light (He et al., 2002; Froehlich et al., 2002; Lee et al., 2003), our demonstration of light responses of the LMO in the absence of *wc-1* supports the idea that a separate light input pathway to the LMO exists (Figure 16). Furthermore, light entrainment in a Δ*wc-1* mutant strain indicates that a different photoreceptor is involved in light signaling to the LMO.

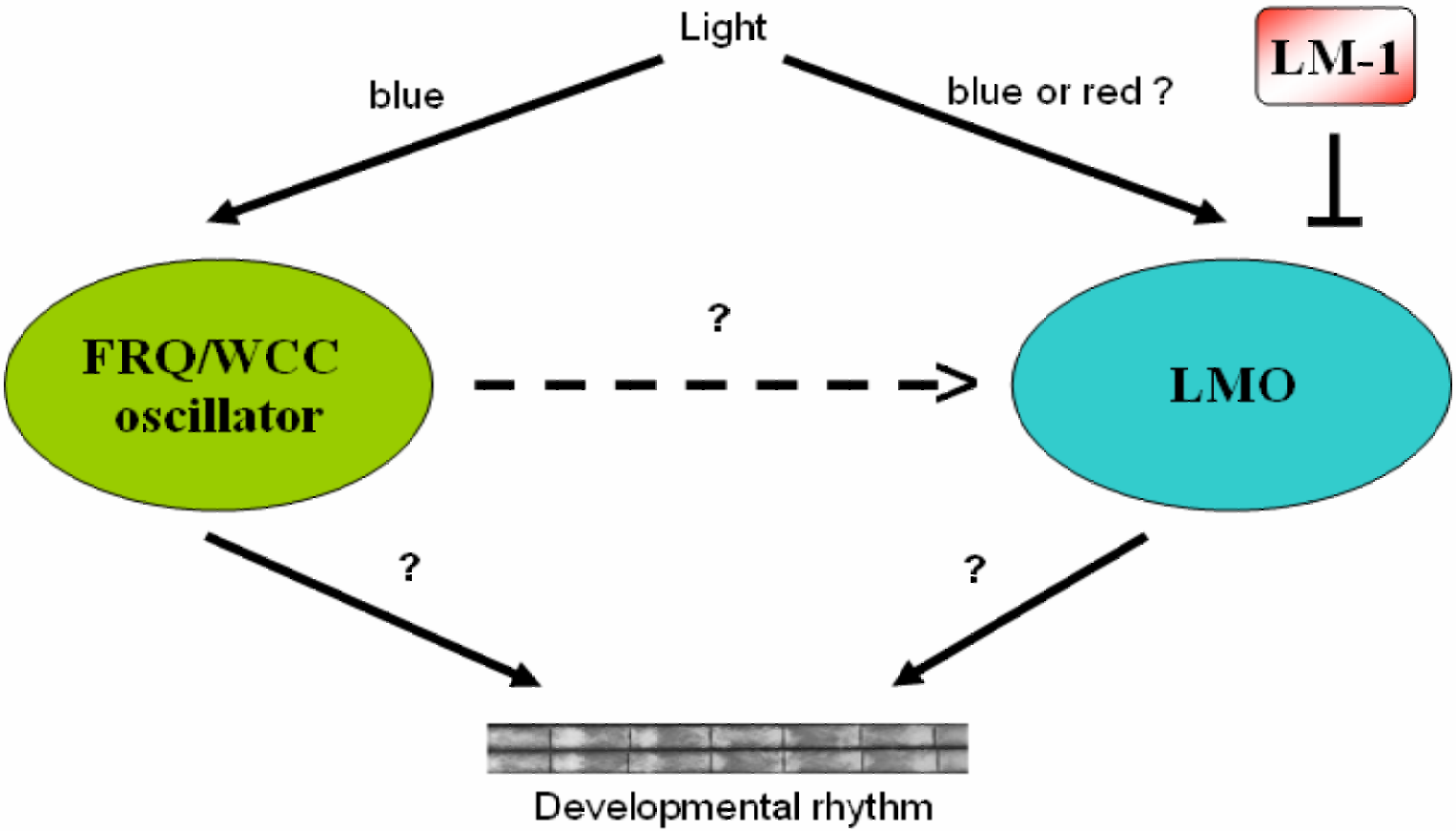


Figure 16. A Simplified Model of the Circadian Oscillators in *N. crassa*. Arrow represents the information flow. ⊣ represents repression. – – represents connection. ? represents unknown function. See the text for details of the model.

VIVID (VVD) is a candidate photoreceptor which may be involved in light signaling to the LMO. VVD is a small PAS/LOV domain-containing protein which is most similar to the PAS/LOV domain of WC-1 (Heintzen *et al.*, 2001). VVD functions as blue light photoreceptor in *N. crassa*. WC-1 is required for the expression of VVD (Cheng *et al.*, 2003a, Schwerdtfeger and Linden, 2001 and Shrode *et al.*, 2001). The VVD feedback loop mutes light responses in *N. crassa* and regulates light resetting and photoentrainment of the circadian clock (Heintzen *et al.,* 2001 and Schwerdtfeger and Linden, 2003). A number of potential light signaling proteins with similarities to phytochromes (PHY-1 and PHY-2), cryptochrome (CRY), opsin (NOP-1), or LOV domain-containing protein (PHOT) have also been found through mining the *N. crassa* genome sequence (Froehlich *et al.*, 2005). Thus, other blue and red light signaling pathways exist in *N. crassa*, and they may be involved in light signaling to the LMO. The role of the candidate photoreceptors in entrainment of the LMO can first be narrowed down by examining light entrainment using light of only the blue and red wavelength. Different light filters will be added to the white light bulbs which were used in these studies before to assay for light entrainment to distinguish these possibilities. Once we establish the type of photoreceptors used by the LMO, the photoreceptor can be identified using available knock-outs of the genes. These knock-out mutant strains need to be crossed to the *lm1 Δwc-1* mutant strain to generate triple mutants to be assayed for light entrainment. The triple mutant(s) who shows the loss of entrainment will indicates that these potential light signaling proteins might be involved in the light signaling pathway.

The LMO can function independent of the FRQ/WCC oscillator to control developmental rhythms in LL

In LL at 25 °C, DBP 580 (*mat A; bd;* Δ*wc-1*), DBP 287 (*mat a; bd;* Δ*frq*), and DBP 339 (*mat A; bd; frq*^{7}) strains were arrhythmic (Figure 10), while strains carrying the LM-1 mutation in these genetic backgrounds exhibited circadian rhythmicity. This implies that the circadian rhythmicities of the LM-1 mutant strains in LL do not require components of the well-characterized FRQ/WCC oscillator. These results support the idea that the LMO responsible for the developmental rhythms can function independent of the FRQ/WCC oscillator (Figure 16). The existence of the FRQ/WCC oscillator and the LMO may enhance the ability of the circadian clock system in *N. crassa* stability and robustness, and flexibility to the environmental changes.

The FRQ/WCC oscillator and the LMO communicate with each other

While our data demonstrate that the LMO can function independent of the FRQ/WCC oscillator to control developmental rhythms in LL, several lines of evidence suggest that the FRQ/WCC oscillator and the LMO communicate with or influence each other.

First, if the FRQ/WCC oscillator and the LMO communicate with or influence each other, then the periods of the LM-1 mutant in the WT, Δ*wc-1,* Δ*frq* or *frq*^{7} background should be different. If they do not communicate with or influence each other, then the periods in these backgrounds should be the same. My results in LL show the period of the LM-1 mutant is 17.1 h, whereas in the Δ*wc-1* or Δ*frq* background, the period is

Table 4. The LM-1 Mutant Strain in *frq*$^{+}$, Δ*frq* and *frq*^{7} Genetic Backgrounds

	lm1 bd; frq^{+}	*lm1 bd;* Δ*frq*	*lm1 bd; frq*^{7}
LL at 25°C	17.1±1.3 h (n=194), high amplitude	25.0±2.0 h (n=81), high amplitude	13.6±0.7 h (n=172), high amplitude
DD at 25°C	22.8±0.3 h (n=91), high amplitude	21.7±1.7 h (n=34), low amplitude	31.1±0.2 h (n=35), high amplitude

lengthened to about 25 h supporting the idea that the two oscillators communicate (Figure 10 and Table 4). Moreover, in a strain with increased FRQ stability (*lm1 A; bd; frq*^{7}) (Aronson et al., 1994), the period is decreased to 13.6 h (Figure 10 and Table 4). This result is particularly interesting, and suggests that a slower FRQ/WCC oscillator correlates with a faster LMO. It would be very interesting to determine if a short period *frq* mutant, such as *frq*^{1}, which has a 16.5 h period (Aronson et al., 1994) lengthens the LMO. Furthermore, in DD the period length of the LM-1 mutant strain is independent of the presence of FRQ or WC-1, suggesting that the LMO dominates period determination in DD.

In addition, if the FRQ/WCC oscillator and the LMO communicate with or influence each other, then the periods of the LM-1 mutant in LL, conditions in which the FRQ/WCC oscillator is normally not functional, and in DD, conditions in which the FRQ/WCC oscillator is normally functional, should be different. While if they do not communicate with or influence each other, then the periods in LL and DD should be the same. Our results show the period of LM-1 in LL is 17.1 h, whereas in DD the period is 22.8 h (Figure 10 and 10) further supporting a connection between the LMO and FRQ/WCC oscillator. The robustness of the rhythm in DD also correlates with the presence and absence of a FRQ/WCC oscillator, further suggesting linkage between the two oscillators. Replicate experiments are currently in progress to allow statistical analysis of the data.

Preliminary data indicate that both the LM-1 and LM-2 mutations are recessive (Seo, personal communication), indicating that the products of the LM-1 and LM-2 genes are not themselves part of the LMO. These data, along with our genetic analysis of the mutants suggest that the LM-1 and LM-2 gene products function to repress the LMO as negative regulators (Figure 16). We do not know if the LM-1 and LM-2 gene products function on the same LMO, or even within the same pathway. In addition, we do not know if the FRQ/WCC oscillator can function when the LMO is inactivated, or if the LMO feeds back to the FRQ/WCC oscillator to influence its activity. Answers to these and other questions will await the identification of the LMO components and cloning of the LM-1 and LM-2 genes.

4. GENETIC MAPPING OF THE LM-1 MUTATION USING CLEAVED AMPLIFIED POLYMORPHIC SEQUENCE (CAPS) MARKERS

4.1 Introduction

To understand the role of the LM-1 mutation in the function of the LMO and the clock, the LM-1 mutation needs to be mapped and the corresponding gene cloned.

In *N. crassa*, mapping of mutations typically employs co-segregation of phenotypic (e.g. auxotropic) markers (Perkins, 1990). Several crosses are required to gain adequate resolution to identify the affected gene by candidate gene prediction (Kotierk and Smith, 2004). Compared to using phenotypic markers, molecular markers are much more numerous and relatively easy to screen (Jenkins, 2003). Molecular markers can result in denser genetic map with greater accuracy from a single cross.

Polymorphisms, usually single-base differences, exist between the *N. crassa* laboratory standard Oak Ridge WT strain and an exotic Mauriceville WT strain (Metzenberg, 1984). Some of these polymorphisms will form or destroy a restriction endonuclease site relative to the Oak Ridge background. Polymorphisms that alter restriction enzyme recognition sites form the basis of PCR-based Cleaved Amplified Polymorphic Sequence (CAPS) markers. The CAPS markers correspond to a defined genomic region and contain a polymorphic restriction enzyme recognition site. This enables differentiation of the parent of origin. CAPS markers have been identified that are

distributed throughout the *N. crassa* genome (Jin et al., 2006). The intervals between CAPS markers have been examined to ensure complete coverage of the whole genome, and they are placed to enable interpolation of linkage data. Using a CAPS mapping approach, an Oak Ridge strain carrying the desired mutation is crossed to the Mauriceville strain and a modified bulked co-segregation analysis is performed using CAPS markers which differentiate polymorphic differences that exist between the two parental backgrounds.

Bulked segregant analysis is also employed in this approach to improve the efficiency of genetic mapping of monogenic traits (Michelmore et al., 1991). Two pooled DNA samples are prepared from individual progeny of a segregating population according to the trait of interest. Each bulk, or pool, contains progeny which have the same genotypes at the region linked to a particular trait of interest or genomic region, but contain random genotypes at all unlinked regions. Hence, the two bulks are genetically dissimilar in the selected region, but are randomized at all the other areas. CAPS markers positioned near the area of interest will be in linkage disequilibrium and markers located further away will display a level of disequilibrium proportional to their distance. At long distances on the same chromosome, CAPS markers will exhibit as much as 50% recombination, indistinguishable from unlinked loci (Jin et al., 2006). This approach was used to map the LM-1 mutation.

4.2 Materials and Methods

Strains, culture conditions and race tube assay

All strains used in this study are listed in Table 5. All assays are the same as described in Section 2.

Genomic DNA isolation from mycelia

Mycelia were collected from 1 ml cultures after growing at 30 °C for 2 days (Jin et al., 2007). Mycelial pads were rinsed with water and vacuum filtered until just damp, and then transferred to 1.5 ml microcentrifuge tubes, frozen with liquid nitrogen. Mycelia were ground to a fine powder with a pestle and a motar. To each ground sample, 10 ml Extraction Buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1% SDS) and 50 µl RNase A (10 mg/ml in Tris-HCl, pH 8.0 and 50% glycerol) were added, mixed vigorously and the tubes incubated at 37 °C for 20 minutes. 50 µl Proteinase K (20 mg/ml in 20 mM Tris-HCl, pH 7.5 and 50% glycerol) was then added, mixed well and the tubes incubated at 65 °C for 45 minutes. After the 45 minutes incubation, 10 ml phenol/chloroform was added, mixed thoroughly and centrifuged for 10 minutes at 3,000 rpm. The aqueous phase was collected and another 10 ml phenol/chloroform was added to the supernatants. The samples were mixed thoroughly and the tubes were centrifuged for 10 minute at 3,000 rpm again. The aqueous phase was isolated and an equal volume of isopropanol was added to the supernatants to precipitate genomic DNA. The DNA pellets were washed once with 70% ethanol. The air-dried DNA pellets were resuspended with 200 µl TE Buffer, pH 8.0, and 2 µl RNase A.

Table 5. *N. crassa* Strains Used in this Study

Strains	Genotype	Phenotype	Source/Reference
FGSC[a] 1858	*mat A; bd*	wild-type; 22 h period in DD at 25°C	FGSC 1858
DBP[b] 695	*lm1 mat a; bd*	17 h period in LL at 25°C	stored by He Huang
DBP 697	*lm1 mat a; bd; Δwc-1*	25 h period in LL at 25°C	stored by He Huang
DBP 751	Mauriceville *mat A; bd-Hyg*^{R}	wild-type; 22 h period in DD at 25°C	stored by Teresa Lamb

[a] Fungal Genetics Stock Center, University of Missouri, Kansas City, MO. [b] Dr. Deborah Bell-Pedersen lab stock number.

CAPS marker design

In addition to the CAPS markers described (Jin et al., 2007), additional markers for the right arm of linkage group I were designed to permit even higher resolution mapping on chromosome I (Table 6). The sequence differences between Oak Ridge WT strain and Mauriceville WT strain were searched through the *N. crassa* genome database (http:// www.broad.mit.edu/annotation/ genome/neurospora /Home.html). Non-identical restriction enzyme sites existing near the different base pairs were used to generate these CAPS markers. Additional CAPS markers designed by Dr. Jennifer Loros were used to further narrow down the mapped region (Table 6).

PCR conditions, enzyme digestion and gel electrophoresis

A single PCR amplification scheme was used for all of the CAPS primer markers (Jin et al., 2007): 50 ng genomic DNA, 0.5 μM each pair of primers, 0.25 mM dNTPs (Fisher Scientific, Houston, TX), 1 U TaKaRa ExTaq polymerase (Fisher Scientific, Houston, TX) and 1 X ExTaq buffer (Fisher Scientific, Houston, TX) were assembled in a 20 μl total volume on ice. Thermal cycler programs were started and paused when the block temperature reached 95 °C before PCR tubes were inserted. Samples were initially denatured for 3 minutes at 95 °C, then treated with 26 cycles of 15 seconds denature at 95 °C, 15 seconds annealing at 60 °C, 1 minute extension at 72 °C, followed by 5 minutes final extension at 72 °C then stored at 4 °C (Jin et al., 2007). Restriction enzymes are from New England Biolabs (Beverly, MA). The intensity of bands on gels was measured by Image J software (Bethesda, MD).

Table 6. Additional CAPS Markers Used in this Study

Marker	Enzyme	Primers	Location	DNA fragment sizes (bp) in Oak Ridge	DNA fragment sizes (bp) in Mauriceville	Source
1-75	DdeI	5'TTCGATTTCAGCCAC AAGGC3' (forward) 5'TTCTTCTGCTCGTCG ACGAC3' (reverse)	contig 6: 728648-729223	182/395	577	Dr. Xiaoguang Liu and He Huang
1-115	SacII	5'TCCGTTGCCTACAAG A ACGT3' (forward) 5'GTGAGGTTATCCCTG AGGAG3' (reverse)	contig 9: 75146-75381	683	287/396	Dr. Xiaoguang Liu and He Huang
1-130	Tsp509I	5'TGGCTCGATCACTTC TGTTTT3' (forward) 5'GTACGTTAGCATCGA AAATGC3' (reverse)	contig 65: 42893	576	208/368	Dr. Jennifer Loros
1-140	HhaI	5'ATAAGTCGGAGGCA GTGAGC3' (forward) 5'CGCAAGTGACACTA TCGCTATC3' (reverse)	contig 38: 97377	431	200/231	Dr. Jennifer Loros
1-144	Tsp509I	5'CTTCCTCACGGCACA TTCAC3' (forward) 5'ACGTCGAAAAGATG GAATCG3' (reverse)	contig 2: 1697508	579	220/359	Dr. Jennifer Loros
1-146	MspI	5'GAGGGCCATCTTGA GCAC3' (forward) 5'CTTGAAAGCGTGCTT GTGG3' (reverse)	contig 2: 1556616	191/240	431	Dr. Jennifer Loros

4.3 Results

The LM-1 mutant maps to the right arm of chromosome I

To identify the location of the LM-1 mutation in the *N. crassa* genome, DBP 695 (*lm1 mat a; bd*) in the Oak Ridge background was crossed to DBP 751 (*mat A; bd-Hyg*^{R}) in the Mauriceville background (Jin et al., 2006 and Beasley et al., 2006) (Figure 17). Forty progeny with the LM-1 mutant phenotype (rhythmic on race tubes in LL at 25 °C in the Percival incubator) and 40 individual progeny with a WT phenotype (arrhythmic on race tubes in LL at 25 °C in the Percival incubator) were isolated. Genomic DNA was extracted from the mycelia of each individual progeny and pooled. A single PCR amplification method was performed for the set of PCR-based molecular CAPS markers and then cleaved using the appropriate restriction enzymes (Jin et al., 2006). The pool of the LM-1 mutant and the pool of WT were mixed at a 1:1 ratio as the controls (Figure 18). The parents of DBP 695 (*lm1 mat a; bd*) in Oak Ridge background and DBP 751 (*mat A; bd-Hyg*^{R}) in Mauriceville background were also used as controls to compare the band intensities, no DNA controls were also used for each CAPS marker (Figure 18).

From the gel electrophoresis after restriction enzyme digestion, tight linkages of LM-1 to CAPS markers of 1-110 and 1-150 were observed (Figure 18). CAPS markers 1-85, 1-184 and 1-185 showed intermediate linkage to the LM-1 mutation, while all the other CAPS markers showed no linkage to the LM-1 mutation (data not shown). The intensities of bands on the gels were measured by Image J software. The bands of the LM-1 mutant on the gel using CAPS marker of 1-59 exhibited 22% Mauriceville

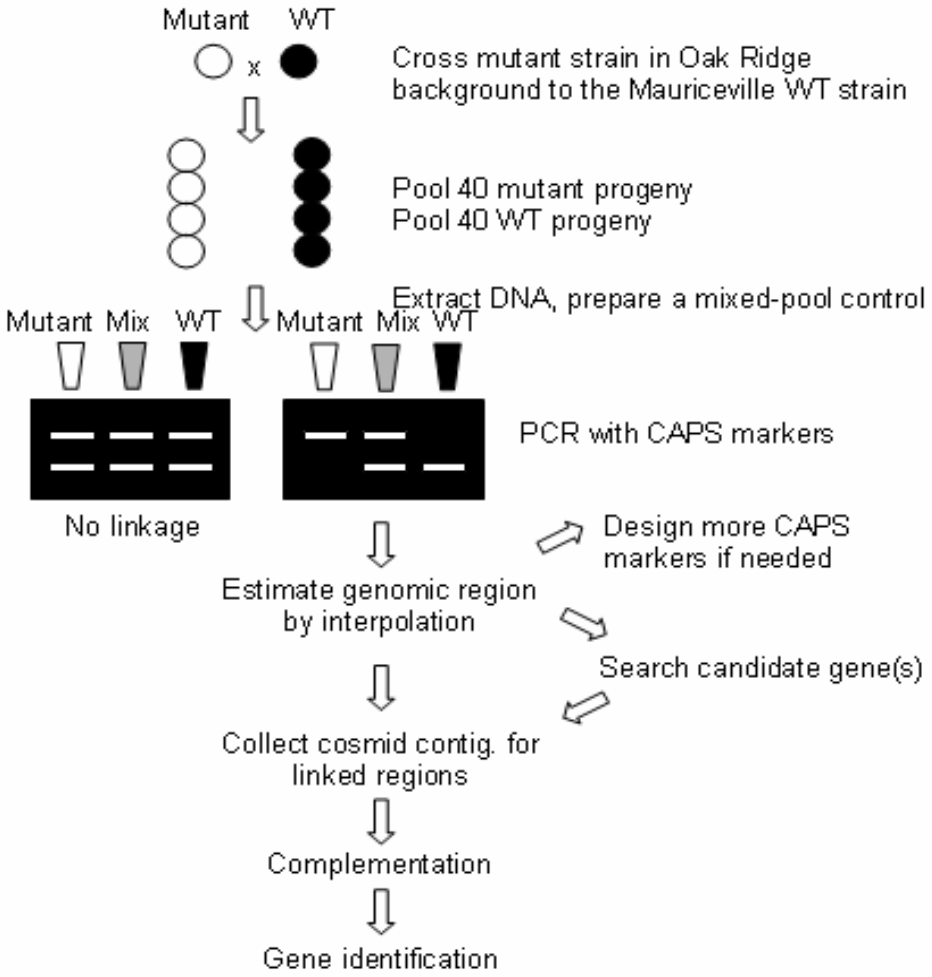


Figure 17. Cartoon Schematic Flow Chart for Map-based Cloning by Bulked Segregant Analysis. (based on Jin et al., 2006)

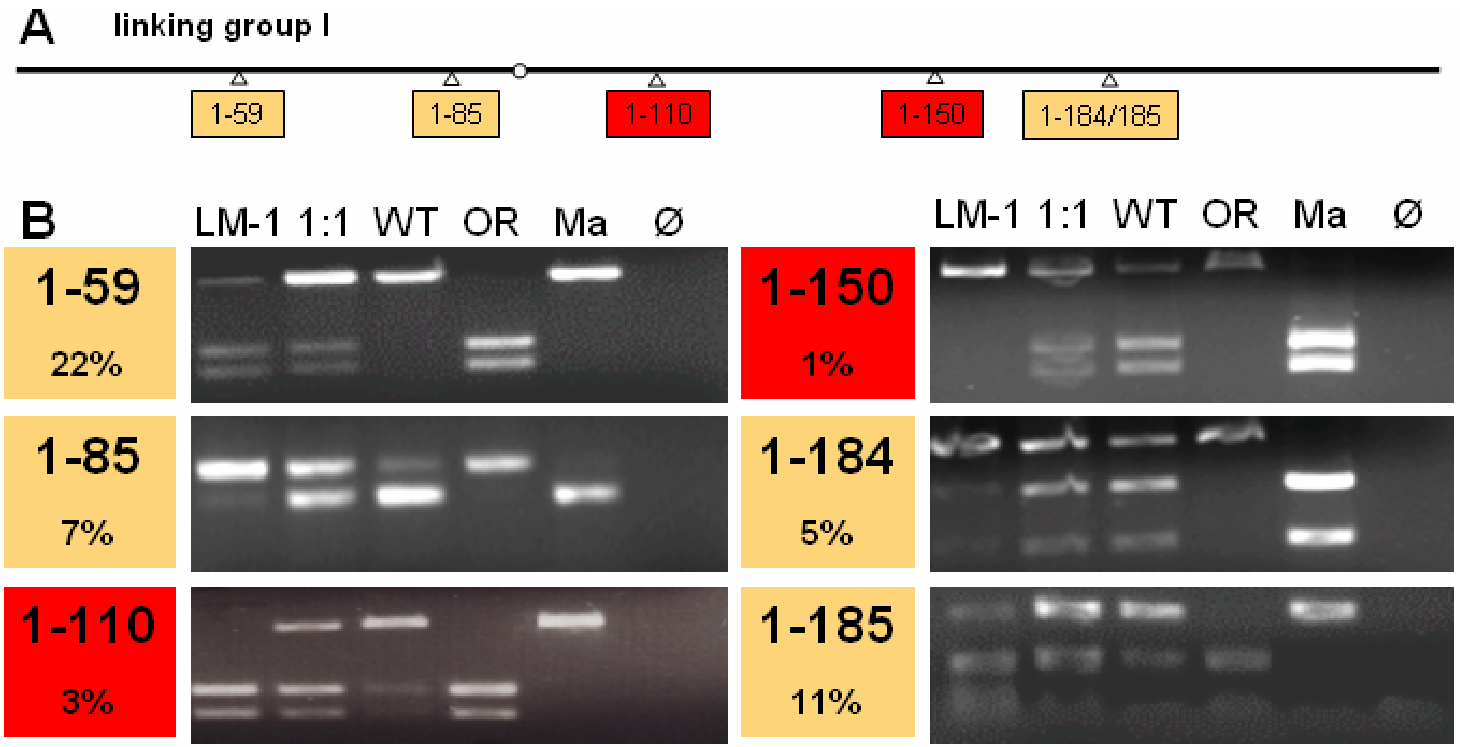


Figure 18. Group Test to Map the LM-1 Mutant by CAPS Markers.
Red boxes represent the recombination rates of equal or less than 3%, yellow boxes represent the recombination rates of more than 5%.

(A) Cartoon schematic of the group test mapping results on the genetic map of linking group I in *N. crassa*. MU represents map unit, MB represents million base pair. ○ represents the centromere. △ points the position of marker.

(B) Argarose gels of linkage results using indicated CAPS markers. The percentages of bands of the LM-1 mutant exhibiting Mauriceville background were present. LM-1 represents pooled DNA from 40 progeny with the LM-1 mutant phenotype. 1:1 represents the mixture of pooled DNA from 40 progeny with the LM-1 mutant phenotype and pooled DNA from 40 progeny with a WT phenotype at a 1:1 ratio. WT represents pooled DNA from 40 progeny with a WT phenotype. OR represents DBP 695 (*lm1 mat a; bd*) as Oak Ridge background control. Ma represents DBP 751 (*mat A; bd-HygR*) as Mauriceville background control. Ø represents no DNA.

background. 7% Mauriceville background was exhibited for CAPS marker of 1-85, 3% for 1-110, 1% for 1-150, 5% for 1-184 and 11% for 1-185.

A map of all CAPS markers tested on linkage group I, along with the corresponding recombination frequencies for selected progeny with the LM-1 mutant phenotype are shown in Figure 19. This approach tested individual progeny with the LM-1 mutant phenotype. The use of individual progeny allows examination of the recombination break points to further pinpoint the location of the LM-1 mutation. Once the linkage to CAPS markers 1-110 and 1-150 was identified, additional CAPS markers were identified to narrow down the location of the LM-1 mutation. Tight linkages of LM-1 to CAPS markers of 1-140, 1-144 and 1-146 all with recombination frequencies of 3%, similar to 1-150 (Figure 19). While 1-59, 1-75, 1-85, 1-110, 1-115, 1-130, 1-184 and 1-185 showed intermediate of linkage to the LM-1 mutation. Individual progeny that showed recombination break points on the right arm of linkage group I were examined in more detail. Analysis of the individual progeny from the cross with respect to recombination break points, suggested that LM-1, present in the Oak Ridge background lies between CAPS markers 1-150 and 1-184.

4.4 Discussion

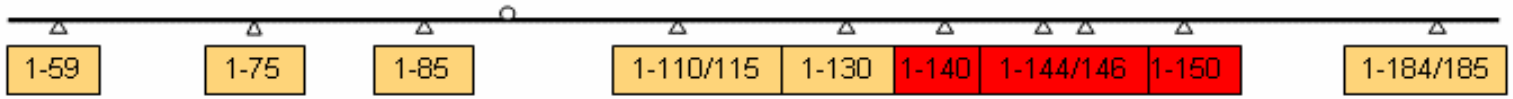
The LM-1 mutant maps to the right arm of chromosome I

Two piece of evidence suggested that the LM-1 mutant maps near the region between the CAPS markers of 1-150 and 1-184.

First, from the pooled DNA group test (Figure 18), the intensity of bands of the LM-1 mutant on the gel using CAPS marker 1-150 shows only 1% Mauriceville background which indicates tight linkage of LM-1 to CAPS marker 1-150. For other CAPS markers on linkage group I, increasing intensities of bands of the LM-1 mutant showing Mauriceville background are observed, indicating intermediate, or no, linkage of LM-1 to those CAPS marker.

Second, from the individual test using progeny with the LM-1 mutant phenotype (Figure 19), only 3% of these progeny exhibited Mauriceville background when the CAPS marker 1-140, 1-144, 1-146 and 1-150 were used, which indicate strong linkage. Other CAPS markers used for this test showed increasing percentages of recombination between LM-1 and the CAPS marker, indicating reduced linkage. Furthermore, recombination break points in individual progeny pinpoint the location of LM-1 between CAPS markers of 1-150 and 1-184, which spans 1069 kb. Additional CAPS markers are being designed to narrow down the genetic region between these two markers.

A linking group I



B

marker / mutant	1-59 12.5%	1-75 11.1%	1-85 12.5%	1-110 8.9%	1-115 6%	1-130 9%	1-140 3%	1-144 3%	1-146 3%	1-150 3%	1-184 12.1%	1-185 12.1%
86	Ma	Ma	Ma	OR	OR	OR	OR	OR	OR	OR	OR	OR
192	Ma	Ma	Ma	OR	OR	Ma	OR	OR	OR	OR	Ma	Ma
210	OR	OR	N/A	OR	OR	OR	OR	OR	OR	OR	Ma	Ma
236	OR	OR	OR	OR	OR	OR	OR	OR	OR	OR	Ma	Ma
238	OR	OR	OR	Ma	OR	OR	OR	OR	OR	OR	OR	OR
275	Ma	N/A	Ma	Ma	Ma	Ma	OR	OR	OR	OR	OR	OR
280	Ma	Ma	Ma	OR	Ma	Ma	Ma	Ma	Ma	Ma	OR	OR
323	N/A	OR	OR	Ma	OR	OR	OR	OR	OR	OR	OR	OR
368	OR	N/A	OR	OR	N/A	OR	OR	OR	OR	OR	Ma	Ma
other 31	OR	OR	OR	OR	OR	N/A	OR	N/A	N/A	OR	OR	OR

Figure 19. Individual Test Using Progeny with the LM-1 Mutant Phenotype to Map the LM-1 Mutant by CAPS Markers.
(A) Cartoon schematic of the individual CAPS markers on the genetic map of linking group I in *N. crassa*. MU represents map unit, MB represents million base pair. ○ represents the centromere. △ points the position of marker. Red boxes represent the recombination rates of equal or less than 3%, yellow boxes represent the recombination rates of equal or more than 6%.
(B) The recombination frequencies are shown for the different mutants using the indicated CAPS markers. Percentages of recombination frequency are present. Ma represents Mauriceville. OR represents Oak Ridge. N/A means not tested.

5. SUMMARY AND CONCLUSIONS

The LMO was unmasked by two recessive mutations (LM-1 and LM-2) in which developmental rhythms were found to persist in strains that lack FRQ or WC-1 in LL, conditions in which the FRQ/WCC oscillator is not functional. The objectives of this project were to determine if the developmental rhythms driven by the LMO are circadian, if components of the LMO communicate with the FRQ/WCC oscillator, and to define the molecular nature of the LMO first by cloning the LM-1 and LM-2 genes.

Our results demonstrated that the LMO displayed the three properties that define a circadian oscillator, including an intrinsic FRP of roughly 24 h when the organism is kept under constant conditions, entrainment by light and temperature, and temperature-compensation.

Consistent with previous data, Δ*wc-1* and Δ*frq* strains do not exhibit developmental rhythms in LD cycles (Aronson et al., 1994a and Lee et al., 2003); however, the LM-1 mutant strain in WT, Δ*wc-1* and Δ*frq* backgrounds is entrained by light (Figures 11 and 12). Since WC-1 is a blue-light photoreceptor required for resetting of the FRQ/WCC oscillator by light (He et al., 2002; Froehlich et al., 2002; Lee et al., 2003), the light entrainment of the LMO in the absence of *wc-1* supports the hypothesis of a separate light input pathway to the LMO. Moreover, because WC-1 encodes a blue-light photoreceptor, light entrainment in a Δ*wc-1* mutant strain indicated a distinct

photoreceptor was involved in light signaling pathway to the LMO. VVD, a blue-light receptor regulating light responses and photoentrainment of the clock in *N. crassa*., is a candidate photoreceptor which may be involved. Other blue (cryptochrome) and red (phytochromes) light signaling pathways, which exist in *N. crassa*, may also be involved in light signaling to the LMO (Froehlich *et al.*, 2005). The role of the candidate photoreceptors in entrainment of the LMO can first be narrowed down by examining light entrainment using light of only the blue and red wavelength. Once we establish the type of photoreceptors used by the LMO, the photoreceptor can be identified using available knock-outs of the genes.

Interestingly, while the LMO can function independent of the FRQ/WCC oscillator to control developmental rhythms in LL, mutations that affect the FRQ/WCC oscillator affect the period of the LMO rhythm in LL. These data suggest that the FRQ/WCC oscillator and the LMO communicate with each other (Figure 16). How this communication occurs is unknown. However, we predict that the FRQ/WCC oscillator and the LMO are not connected through the product of the gene specified by the LM-1 mutation from two pieces of evidence. First, in LL where the FRQ/WCC oscillator is not functional, DBP694 (*lm1 A; bd*) shows developmental rhythm period of 17.1±1.3 h, while DBP833 (*lm1 A; bd; frq*^{7}) exhibits a reduced period of 13.6±0.7 h (Table 4 and Figure 10). Preliminary data indicate that the LM-1 mutation is recessive suggesting loss of activity (Seo, personal communication). Thus, the product of the LM-1 gene is likely not involved in the LMO itself or in the communication between the LMO and the

FRQ/WCC oscillator since the absence of the FRQ/WCC oscillator can influence the period of the rhythm. Similar in DD, where the FRQ/WCC oscillator is functional, DBP694 (*lm1 A; bd*) shows developmental rhythm period of 22.8±0.3 h, while DBP833 (*lm1 A; bd; frq*^{7}) exhibits a long period of 31.1±0.2 h (Table 4 and Figure 10). As in LL, the period lengthening of the developmental rhythm occurs in the absence of the LM-1 gene product. Interestingly in WT strains in LL, the developmental rhythm is lost. This suggests that in LL both the FRQ/WCC oscillator and the LMO are not functional. These data suggest that light increases the activity of the LM-1 gene product. Both the FRQ/WCC oscillator and the LMO may involve in the development pathway. However, it is also possible that the FRQ/WCC oscillator sends information to the LMO which in turn regulate the developmental rhythm.

Using CAPS markers, the LM-1 mutation was genetically mapped to the right arm of linkage group I near the region between CAPS markers 1-150 and 1-184, which spans 1069 kb. Additional CAPS markers are being designed to further narrow down the genetic region. Cosmids which cover the mapped genomic regions where the linked CAPS markers exist are currently being used to test for complementation of the LM-1 mutant phenotype (arrhythmicity in LL). To identify the genes specified by the LM-1 mutation, candidate genes will be cloned individually and introduced into the appropriate LM-1 mutant strain to test for complementation. Once the gene specified by the LM-1 mutation is identified, the LM-1 lesion will be sequenced in the LM-1 mutant strain.

To identify components in the LMO, genetic suppressor screens will be performed. This screen will target components in the LMO through identifying bypass suppressors which abolish or alter the LM-1 rhythms in LL at 25 °C. Mutations in genes which alter the rhythms in LM-1 strain in LL that also lack a functional FRQ/WCC oscillator will be predicted to identify components in the LMO. In order to identify bypass suppressors, strains which overexpress *quelling-defective-1* (*qde-1*), which is a rate-limiting factor in post-transcriptional gene silencing in *N. crassa* will be utilized (Forrest et al., 2004). *lm1 frq*^{9}*,* Δ*qde-1* mutant strain will be transformed with a plasmid that overexpresses QDE-1; over-expression of QDE-1 leads to almost 100% gene silencing when two copies of a gene are present in cells (Fulci and Macino, 2007). In order to eliminate any silencing of *qde-1* expressed from the plasmid, the Δ*qde-1* allele is used. To select hygromycin-resistant cDNA transformants, the *frq*^{9} allele, which is a point mutation and null allele of *frq* will be used (rather than *frq*^{10}, which is hyg^{R}) (Aronson et al., 1994a). The phenotype of *lm1 frq*^{9} strain is identical to *lm1* Δ*frq* strain (Seo, personal communication). This strain will be transformed with a *N. crassa* expression cDNA library available from the FGSC which confers resistance to hygromycin and screened for arrhythmic hygromycin-resistant strains in LL. Each transformant should be silenced with respect to expression of both the cDNA it carries and its corresponding genetic allele. Through this method, we hope to identify the silenced candidate gene in a transformant which has the desired phenotype by retrieving the transforming plasmid. We are currently testing this system using cloned genes that when deleted give observable growth phenotype. A back-up

approach to identify components in the LMO is to use the yeast two-hybrid assay, or immunoprecipitation assay, with LM-1 to identify components of the cell which interact with the LM-1 protein.

Once suppressors of the LM-1 phenotype are identified, a knock-out mutation of the candidate genes will be generated using homologous gene replacement both in the LM-1 mutant and WT strains. This will allow us to determine if knock-out of the LMO affects circadian rhythmicity in an otherwise WT strain, and will provide a test of the model for coupling between the LMO and the FRQ/WCC oscillator (Figure 16).

Circadian clocks are composed of multiple oscillators in diverse organisms, such as cyanobacteria, fungi, algae, plants, flies, birds and man (reviewed by Bell-Pedersen et al., 2005). The prevailing perception in vertebrates has been that the circadian clocks represent cellular processes in brains receive and process external light signals and direct overt rhythmicity in otherwise passive recipient peripheral tissues. The suprachiasmatic nucleus (SCN) of the hypothalamus in mammals has been revealed to be a circadian pacemaker, which is an oscillator that drives rhythmic outputs and/or entrains another oscillator, and can be entrained by light. Therefore, only SCN neurons were initially considered of containing autonomous oscillators which exhibit rhythms in clock genes. However, this view has changed significantly in the past couple of years due to the discovery of autonomous oscillators in tissues and organs which are not connected with neuronal structures. Rhythmicities in the core clock gene expression have been observed

in retina, heart, lungs, kidneys, liver, and even in immortalized cell lines in culture (Shearman et al., 1997; Yamazaki et al., 2000; Zylka et al., 1998). Thus, peripheral tissues also have inherent circadian properties and that regulate tissue-specific outputs in mammals. The results of the LMO, a novel circadian oscillator in *N. crassa*, obtained from this work also provide understanding of the complexity of oscillators in organisms.

Together, the results from my studies suggested a model where the circadian clock system in *N. crassa* is composed of multiple circadian oscillators. In certain genetic background, we have shown that the LMO can function independently to control the developmental rhythms. However, these circadian oscillators communicate with each other to coordinately control overt rhythms in *N. crassa.* These results lead to a better understanding of the complexity of oscillators that form a circadian clock in organisms.

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