Temporal expression patterns of *period* in *cry*^{b} and *vg* mutants of *Drosophila melanogaster*

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In *Drosophila melanogaster,* the gene products of *period* (*per*) and *timeless* are essential components of the circadian clock. The temporal expression patterns of *per* at various time points were studied in the intestine and salivary glands of wild type (WT), *cryptochrome*-absent (*cry*^{b}), and *vestigial* (*vg*) mutants under 12 : 12 h light : dark and 12 : 12 h blue light (450 nm) : dark conditions. The expression of *per* in the tissues closely resembled its expression pattern in brain tissues reported by various authors. At ZT 06 and ZT 10, the expression was almost nil and at ZT 18 and ZT 22, the expression was most pronounced in WT and mutants when compared to other time points. The weaker expression of *per* in *cry*^{b} flies suggested the significant role of blue light photoreceptor, *cryptochrome* for a stronger synchronization of the circadian clock. As *vg* flies have greatly reduced wings, their gross locomotor activity was poorer and levels of *per* expression were also least than WT flies. The expression patterns of *per* in the salivary gland of larvae further suggested the presence of peripheral oscillators during the developmental stages of *Drosophila*.

A wide variety of biochemical, physiological and endocrinological functions in almost all organisms show rhythmic variations in parallel with the day–night (light–dark) cycles. The biological processes controlled by endogenous oscillators or circadian clocks[1] range from daily sleep/wake cycle and levels of various enzymes/hormones to DNA synthesis and cell division. Extensive studies have shown that these circadian (L. *circa*, about: *dies*, a day) rhythms indeed have a genetic basis and are largely cell autonomous[2,3].

In *Drosophila melanogaster*, genetic and molecular analysis showed that several clock genes are necessary for the generation and regulation of overt oscillations. They are: *period* (*per*), *timeless* (*tim*), *clock* (*clk*), *cycle* (*cyc*), *double-time* (*dbt*), etc. The mRNA and protein levels of *per* and *tim* cycle with a $\simeq$ 24 h period throughout the body of *Drosophila*[4,5]. The cyclic regulation of *per* and *tim* and the manner in which they generate a molecular clock have been reviewed extensively[6–8].

Lateral neurons in the brain appeared to be important for circadian regulation in the fly; brain-independent circadian oscillations have been detected in many peripheral tissues of *Drosophila*[2,3,9,10]. Plautz *et al.*[2], demonstrated the cyclic appearance, disappearance and reappearance of *per* expression in the legs, wings, thorax, head and abdomen of the fly. Furthermore, even cultured cells and tissues could be entrained by light–dark cycles, indicating that non-neuronal *Drosophila* cells are photoreceptive and capable of supporting their own independent oscillations[11–13].

Drosophila utilizes at least three photoreceptors for entrainment, viz. the blue light photoreceptor-*cryptochrome*[14–16], the compound eyes (ocelli) and the Hafbauer–Buchner eyelet[17], and all these structures express opsin-based photopigments[18]. These photoreceptors mainly convey the environmental signals to the clock. The clock appears to work from the first instar larvae onwards in *Drosophila*[19,20]; *per* and *tim* are expressed cyclically in larval central nervous system and daily oscillations of *per* expression persist throughout metamorphosis in lateral neurons of larvae[21]. However, the temporal expression patterns of *per* in the intestine of adults and salivary glands of third instar larvae of wild type, *cry*^{b} and *vg* mutants have not been studied.

As photopigment *cryptochrome* (*cry*) is an indispensable component for entrainment in peripheral tissues[9,10,14–16,22], and since *cry*^{b} mutants have defective photic input to the clock[9,10,23,24], it would be of interest to study the expression patterns in the intestine and salivary gland. In *vg* mutant, the wings are much reduced[25] and therefore locomotor activity was poorer in this mutant[26]. Hence it would also be of interest to investigate whether this defective locomotor behaviour is reflected in the expression patterns of *per* gene in the above mentioned tissues under 12 : 12 h light : dark (LD) and 12 : 12 h blue light (450 nm) : dark (BD) treatment conditions.

Cultures of *D. melanogaster* (wild type – Oregon R$^+$, *cry*^{b} and *vg* mutants) were reared on a standard medium containing agar, yeast, maize powder, sucrose and antifungal agent methyl-*p*-hydroxy benzoate under LD and BD conditions in ventilated light-tight boxes (60 × 30 × 30 cm) at 20 ± 2°C. Incandescent bulbs (15 W) were used during the L-phase (300 lux) and 450 nm light regime was maintained using Kodak GBX filter.

Clone of *per* cDNA was amplified in DH5α *Escherichia coli* cells and cDNA was separated from the vector by restriction enzyme (*Hin*dIII) digestion. The cDNA of *per* was eluted (2.231 kb) from low melting agorose gel, purified and then labelled with dogoxigenin-11-dUTP (Roche Diagnostics, Germany) by random primed DNA labelling method. The efficiency of labelling was checked following standard protocols[27].

Adult flies and late third instar larvae of wild type (WT) and mutants (*cry*^{b} and *vg*) were dissected at six different time points in phosphate-buffered saline (ZT 02, ZT 06, ZT 10, ZT 14, ZT 18, ZT 22 and ZT 02; where ZT 00 – lights on and ZT 12 – lights off). The tissues were then subjected to whole mount RNA–DNA *in situ* hybridization. The tissues of intestine from adult and salivary gland of third instar larvae were first fixed with paraformaldehyde and then

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treated with diethylpyrocarbonate (0.1%) and digested with proteinase K. Hybridization of *per* mRNA with *per* cDNA probe (denatured) was carried out at 58–65°C for 24 h period. The unhybridized probes were washed-off with the hybridization buffer. The expression signals of *per* mRNA were identified by incubating the tissues with anti-digoxigenin-AP-fab fragments (Roche Diagnostics, Germany) and chromogenic substrate. A ribonuclease treated (30 min) control at ZT 18 (in which maximum expression was observed) was carried out in the intestine and salivary gland.

Figures 1 and 2 show the temporal expression of *per* in the intestine of adults and salivary gland of third instar larvae of WT, cry^b and *vg* mutants under LD cycle. Figures 3 and 4 show the expression of *per* in intestine of adults and salivary gland of third instar larvae of WT and mutants under BD cycle. Figure 5 shows the ribonuclease-treated controls, showing nil expression of *per* in intestine and salivary gland. The temporal expression patterns are tabulated (Tables 1 and 2); (+) represents *per* expression and (–) represents absence of *per* expression. More number of (+) indicates higher level of expression. In general, in cry^b mutants, the expression was less intensive than *vg* mutants, which in turn is less intensive than WT. At ZT 06 and ZT 10, the expression was almost nil in both the tissues (intestine and salivary gland). At ZT 18, the expression was most pronounced in WT and mutants when compared to other time points. A definite temporal pattern in the levels of *per* expression could also be observed in ZT 02, ZT 14 and ZT 22. Similar types of expression patterns were seen both in intestine and salivary gland.

Rhythmic gene expression is a key mechanism by which the circadian clock regulates physiological and behavioural processes in animals, plants and microbial systems. We have monitored the levels of *per* in intestine of adults and salivary gland of third instar larvae under different light responses (LD and BD conditions). The expression of *per* in the tissues of WT resembled the expression patterns in brain tissues reported by various authors. Light provides essential phase information for all circadian rhythmicity and autonomous photosensitive peripheral clocks are present in a variety of peripheral tissues, as reported by various authors[4,11,12]. Temporal pattern of *per* expression in intestine and salivary gland (Figures 1 and 2) suggests the presence of peripheral oscillators in these tissues.

The stronger expression patterns in WT than mutants (*vg* and cry^b) indicate the lesser coupling and coordination of peripheral oscillators with the master oscillator in mutants. At ZT 06 and ZT 10, almost nil expression of *per* indicates

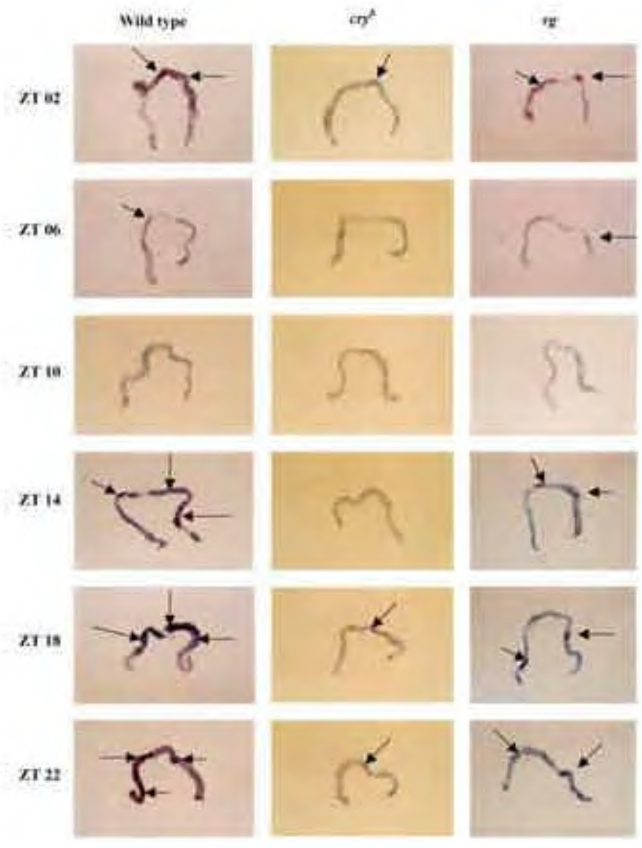

Figure 1. Temporal expression pattern of *per* in intestine of adult flies of WT, cry^b and *vg* mutants under 12 : 12 h light : dark (LD) conditions. Arrows indicate expression sites.

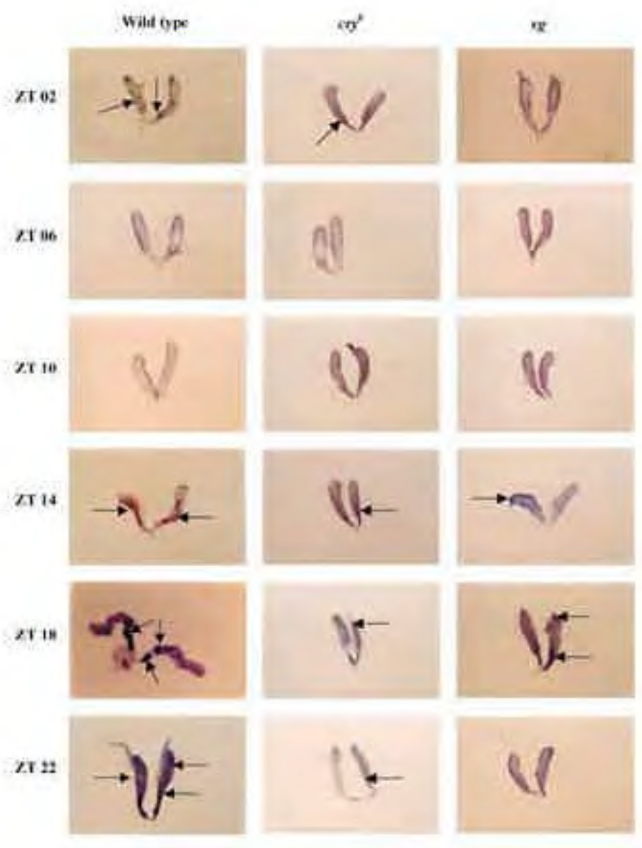

Figure 2. Temporal expression of *per* in salivary gland of third instar larvae of WT, cry^b and *vg* mutants under LD conditions. Arrows indicate expression sites.

Table 1. Temporal patterns of levels of *per* expression under 12 : 12 h light : dark (LD) conditions

	Wild type		cry^b		*vg*	
Zeitgeber time (ZT)	Intestine	Salivary gland	Intestine	Salivary gland	Intestine	Salivary gland
ZT 02	++	++	+	+	++	–
ZT 06	+	–	–	–	+	–
ZT 10	–	–	–	–	–	–
ZT 14	+++	++	–	+	++	++
ZT 18	++++	++++	+	+	+++	+++
ZT 22	+++	+++	+	+	++	–

Table 2. Temporal patterns of levels of *per* expression under 12 : 12 h blue light (450 nm) : dark (BD) conditions

	Wild type		cry^b		*vg*	
Zeitgeber time (ZT)	Intestine	Salivary gland	Intestine	Salivary gland	Intestine	Salivary gland
ZT 02	++	+	–	++	+	+
ZT 06	–	–	–	+	–	–
ZT 10	+	++	–	+	–	–
ZT 14	++	++	+++	+	+	+
ZT 18	++++	+++	++	+	+++	++
ZT 22	++	+	+	–	+	+

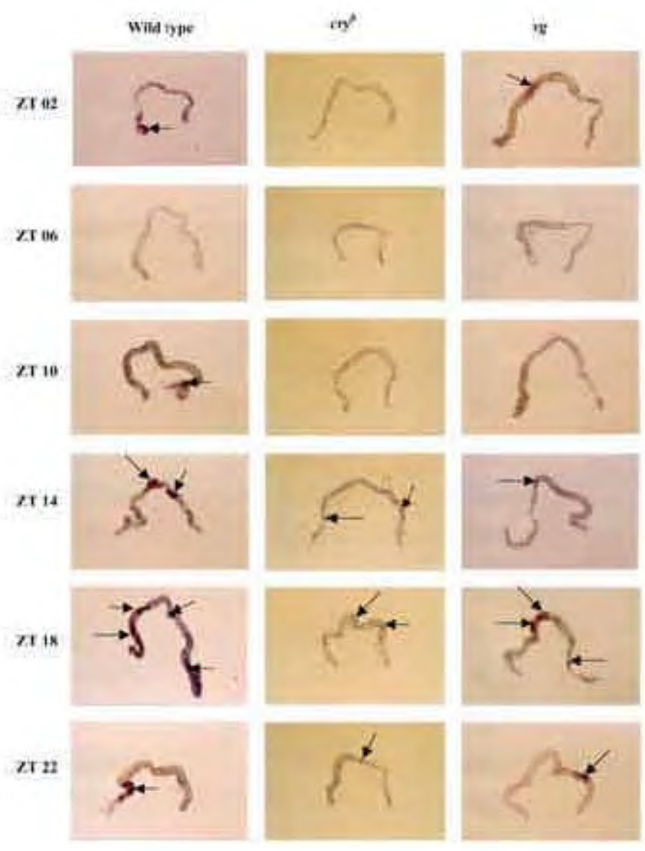

Figure 3. Temporal expression pattern of *per* in intestine of adult flies of WT, cry^b and *vg* mutants under 12 : 12 h blue light (450 nm) : dark (BD) conditions. Arrows indicate expression sites.

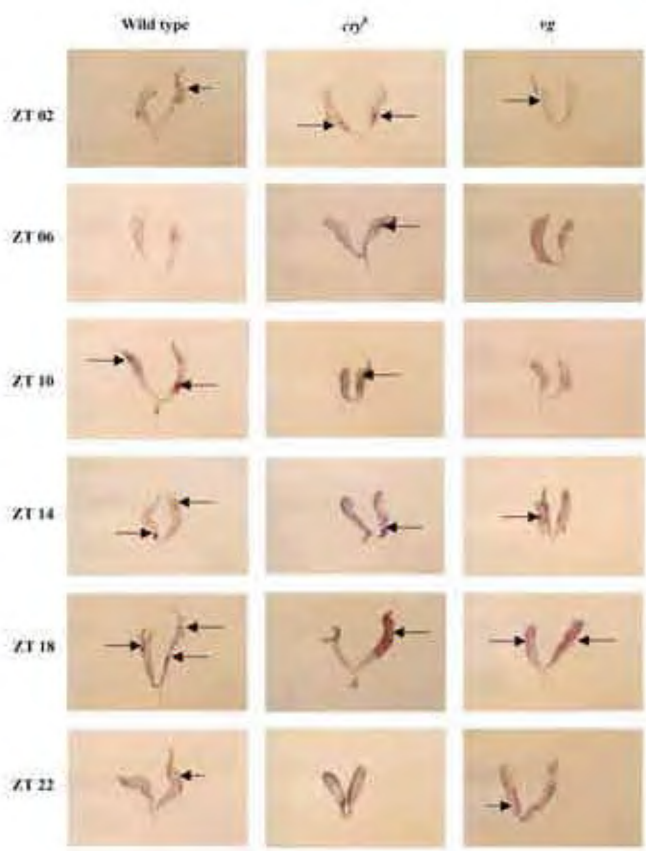

Figure 4. Temporal expression pattern of *per* in salivary gland of third instar larvae of WT, cry^b and *vg* mutants under BD conditions. Arrows indicate expression sites.

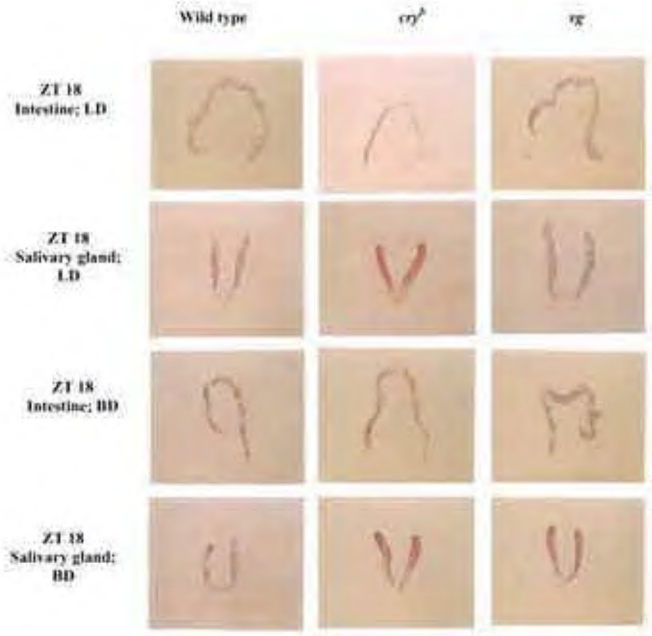

Figure 5. Ribonuclease-treated controls (intestine and salivary gland) of WT, cry^b and *vg* mutants under LD and BD conditions.

that the light-dependant degradation of TIM protein is foolproof. Klarsfeld *et al.*[14] showed that *cry* is an indispensable element for entrainment in peripheral tissues; weaker expression of *per* in cry^b mutants observed in the present study indicates the significant role of blue-light component for stronger synchronization of circadian clock in the fly[3,10]. However, under BD cycle, *per* expression was more than that of LD cycle, even though cry^b flies have defective photic input to the clock. The blue light thus, could mimic darkness in cry^b flies, thereby the levels of *tim* could increase and also coupling with *per*, as increased *per* mRNA levels were seen.

per gene is a central component of the circadian oscillator that controls locomotor activity of the fly[28,29]. In *vg* mutants, we observed that locomotor activity was poorly synchronized[26] and *per* expression was weaker compared to WT. The expression of *per* in the brain of larvae was reported earlier[30] and the clock appears to work from the first instar larvae onwards[19,20]. The present communication elucidates the expression of clock gene in the salivary gland of third instar larvae in WT and mutants, suggesting the presence of peripheral oscillators during the developmental stages of *D. melanogaster*.

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Genetic diversity of Indian isolates of rice blast pathogen (*Magnaporthe grisea*) using molecular markers

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***Magnaporthe grisea*, the rice blast fungus is one of the main pathological threats to rice crop worldwide. The genetic relatedness and the probable mechanisms of genetic variation among the Indian isolates of rice blast pathogen were studied. A total of 171 polymorphic markers were scored using 33 selected random decamer primers. The isolates exhibited an overall polymorphism of about 64%. The similarity degree value for the isolates ranged from 0.76 to 0.92. The high polymorphism could be explained by natural and stress-induced transposition and horizontal gene transfer. Understanding the source of pathogen variation will aid in designing improved methods for management of the rice blast disease.**

MAGNAPORTHE grisea (anamorph: *Pyricularia oryzae*), a filamentous ascomycetes fungus, parasitizes over 50 grasses, including economically important crops like wheat, rice, barley and millet[1]. But the pathogen is best known as the casual agent of the rice blast disease. Rice is an important agricultural crop supplying approximately 23% of the per capita energy for six billion people worldwide[2]. Rice blast is the most serious disease in all rice-growing regions of the world. Under heavy dew, all aerial parts of the plant can be affected; leaf surfaces become speckled with oval lesions, plants are liable to lodging if stems are infected. If the panicle is infected, then a severe yield loss results[1]. The fungus has the ability to overcome resistance within a short time after the release of a resistant cultivar and thus has made breeding for resistance a constant challenge. The analysis of genetic variation in plant pathogen populations is an important prerequisite for understanding coevolution in the plant pathosystem[3]. Populations of rice blast pathogen throughout the world have been studied for their phenotypic and genetic variation[4–8]. Although earlier studies focused on pathotypic variability[1], recent studies utilized molecular markers to characterize population diversity. Extensive use of the MGR586 heterodispersed element[8–11] to delineate DNA fingerprint lineages has helped to clarify the genetic structure of this important pathogen.

Polymerase chain reaction (PCR)-based molecular markers are useful tools for detecting genetic variation within populations of phytopathogens[12,13]. Random amplified polymorphic DNA (RAPD)[14,15] markers have been widely used for estimating genetic diversity in natural populations[16], mainly because the technique does not need previous molecular genetic information and increases marker density for evaluating genetic relationship. The RAPD technique has also been used to study genetic diversity among rice blast pathogens from Portugal[17]. The objectives of the present investigation were to study the genetic variability among isolates of *M. grisea* from different geographical regions.

M. grisea isolates were revived and grown on potato dextrose agar (PDA, Hi Media) plates at 25°C for 5 days (Table 1). For DNA extraction, isolates were grown in 100 ml of potato dextrose broth for 4 days at 25°C in a rotary shaker at 100 rpm. Mycelial mat was filtered, dried and ground to a fine powder in liquid nitrogen. Powdered mycelia were vortexed in pre-warmed lysis buffer [100 mM Tris (pH 8.5), 250 mM NaCl, 0.5 mM EDTA and 0.5% SDS], incubated at 65°C for 30 min followed by the addition of 1.7 M potassium acetate solution. The contents were gently mixed and incubated on ice for 30 min. Samples were then extracted with chloroform and the total nucleic acid was precipitated with chilled isopropanol. The pellet after centrifugation and drying was dissolved in TE (10 mM Tris and 1 mM EDTA, pH 8.0). After RNAase treatment, the DNA was purified with phenol : chloroform (1 : 1; v/v) and chloroform : isoamylalcohol (24 : 1; v/v) and precipitated with chilled ethanol after adding 1/10th volume of 3 M sodium acetate. The DNA was dissolved in TE buffer. The DNA concentration was estimated with a DNA fluorometer (Hoefer Scientific, San Francisco, USA) using Hoechst 33258 and calf thymus DNA[18].

Primer survey was carried out using random decamer primers from kits A, F, G, K, L, M and N (Operon Technologies, Almeda, USA). A total of 128 RAPD primers were screened using DNA from three isolates, namely Maruteru, Almora and Karjat CV4. Thirty-three primers that gave

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