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Journal of Insect Physiology 51 (2005) 655–659
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Is *period* gene causally involved in the photoperiodic regulation of reproductive diapause in the linden bug, *Pyrrhocoris apterus*?

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Received 15 November 2004; received in revised form 10 January 2005; accepted 10 January 2005

Abstract

Earlier experiments demonstrated a strong up-regulation of *per* mRNA in wild-type (Wt) females of *Pyrrhocoris apterus* reared under diapause-inducing short days, while *per* mRNA levels were low in females of two non-diapause mutant strains (Nd), irrespective of photoperiod. In the present study, different sequences of *per* DNA in two strains of geographically different origin enabled us to analyse genetic linkage between the *per* gene and the Nd phenotype. Crosses between Wt females originating from C. Budejovice (Czech Republic) and Nd males originating from Lyon (France) resulted in F_2 progeny where 411 females entered diapause under short days and 120 females were reproducing. Thus, the segregation was very close to the 3:1 ratio in favour of diapause females, suggesting that the Nd trait behaves as a single autosomal recessive. Analysis of DNA in 20 females of the F_2 progeny revealed that their phenotype was not linked to the *per* genotype. We conclude that the *per* gene is not primarily responsible for the block to diapause photoresponsiveness in Nd mutants and its role, if any, is downstream from other gene(s) controlling diapause. This is the first attempt at genetic linkage analysis between a bona fide circadian clock gene and photoperiodism in a 'non-drosophilid' species.

Keywords: Circadian clock; *Period* gene; Genetic linkage; Photoperiodism; Diapause

1. Introduction

It is generally accepted that circadian oscillators are involved in photoperiodism but molecular mechanisms are poorly understood (Denlinger, 2002; Saunders, 2002). In recent years, a potential role of circadian clock genes in photoperiodism has been the subject of extensive discussions (Tauber and Kyriacou, 2001; Saunders et al., 2004).

The linden-bug, *Pyrrhocoris apterus*, shows reproductive diapause induced by short days. Under short days, the level of *period* mRNA in the insect head is about 10-fold higher than under diapause-preventing long days. In mutant insects that do not undergo diapause, even under short days, levels of the transcript are low under both photoperiods. The differential regulation of *per* gene transcripts was demonstrated in two independently selected mutant strains, one originating from Ceske Budejovice (Czech Republic) (Hodkova et al., 2003; Syrova et al., 2003) and one from Lyon (Dolezel, unpubl.). These results strongly indicate that the *per* gene is implicated in the photoperiodic induction of diapause in *P. apterus*. In seasonal mammals, the amplitude of *Per 1* gene expression is suggested to be part of the mechanism through which photoperiodic time is decoded in the pars tuberalis of the pituitary (Messager et al., 1999, 2000) and in other tissues (Carr et al., 2003). On the other hand, a causal relationship between the *per* gene and photoperiodic induction of ovarian diapause have been excluded in *Drosophila melanogaster* (Saunders, 1990).

Genetic analysis, including reciprocal crosses and backcrosses between non-diapause mutants and a

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0022-1910/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2005.01.009

wild-type strain of *P. apterus* revealed that the non-diapause trait behaves as a single autosomal recessive (Socha and Hodkova, 1994). The aim of the present study was to determine whether the non-diapause trait is genetically linked to the *per* gene itself.

2. Material and methods

2.1. Insects

Adults of *P. apterus* (L.) (Heteroptera) were used in all experiments. Insects were reared at 26±2 °C and supplied ad libitum with linden seed and water. The wild-type strain (Wt) originated from adults collected from the field near Ceske Budejovice, Czech Republic, and was maintained under a diapause-preventing long-day photoperiod of 18 h light/6 h darkness (LD). Two mutant strains (Nd) lacking the diapause response were selected under a diapause-promoting short-day photoperiod of 12 h light/12 h darkness (SD) and 26 °C from a few reproducing individuals (Hodkova and Socha, 1992). One mutant strain originated from the same locality as wild-type insects, the other from a field near Lyon, France.

For genetic linkage analysis, crosses were made between Wt females and Nd Lyon males. Several single pair matings in Petri dishes were made for each cross. Adult females of F_1 and F_2 generations were kept individually in Petri dishes and evaluated for either diapause (no oviposition) or non-diapause (oviposition) phenotype 3–4 weeks after adult ecdysis. At the end of the experiment, insects were placed individually to −80 °C and kept at this temperature until analysis.

2.2. DNA isolation, PCR, sequencing

Part of the whole body of individual females was mechanically homogenized and genomic DNA was isolated with a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed with ExTaq polymerase (Takara, initial denaturation 92 °C, followed by 30 cycles of 92 °C −20 s, 55 °C −30 s, 72 °C −1 min).

Gene fragments containing strain specific polymorphisms used for linkage analyses were amplified with forward: 5′ ACAGCTAGTGGTGGTGAAGAGG and reverse: 5′ AAAAGTTGTTCAGTAAGAGCAGTAG primers. These PCR products (1126 bp) were gel purified and sequenced with amplification primers (sequencing with forward primer identifies polymorphism I, while the reverse primer identifies polymorphism II).

3. Results

3.1. Strain specific polymorphism

A 3 kb fragment of the *period* gene (corresponding to 371 bp of *per* cDNA) was PCR amplified and the entire fragment was sequenced. This part of the *period* gene contained 3 exons and 2 introns (1535 and 928 bp long) (Fig. 1). No sequence differences were observed between Wt and Nd strains originating from C. Budejovice. On the other hand, several single nucleotide polymorphisms were identified between the Lyon and C. Budejovice Wt strains (Fig. 2). Strain specific *period* gene sequences were deposited in GeneBank (CB-Wt: AY803767, AY803770 and Lyon ND: AY803768, AY803769). Sequence analyses of F_1 progeny confirmed that both males and females were heterozygous for *per* alleles. *P. apterus* belongs to X0-sex determination (Socha, 1993), thus *period* is autosomal. Hence, the *period* from Lyon Nd strain, C. Budejovice Wt strain and their heterozygots can be identified by sequencing. Therefore we decided to use these markers to analyze the genotype of F_2 progeny and test if the *period* gene is linked to the diapause /non-diapause phenotype.

3.2. Genetic linkage analysis

Crosses were made between the Wt females from C. Budejovice and Nd males from Lyon. F_1 progeny were reared at SD from a young larval stage. All F_1 females entered diapause, indicating a recessive character of the non-diapause trait (Table 1). Diapause F_1 females and males were activated by transfer to LD. F_2 progeny were produced by intercrosses between F_1 siblings. Young larvae of F_2 progeny were transferred to SD and females

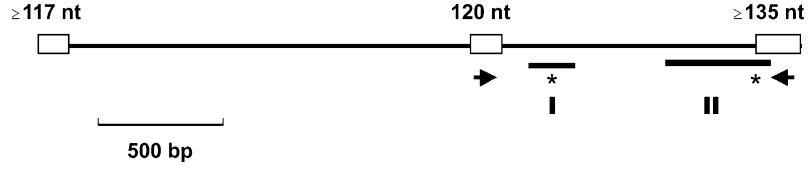

Fig. 1. Structure of *period* gene fragment determined in this study 3 kb genomic region consists of two introns (1535 and 928 bp long) and 3 exons (117, 120 and 135 bp long, please note that marginal exons are not sequenced completely). Arrows indicate primers used for PCR and sequencing. Horizontal bars correspond to sequences deposited in a GeneBank (*I* = *polymorphism* I, AY803767 and AY803768; *II* = *polymorphism* II AY803769 and AY803770) and asterisks indicates position of strain specific polymorphisms (sequence detail is shown in Fig. 2).

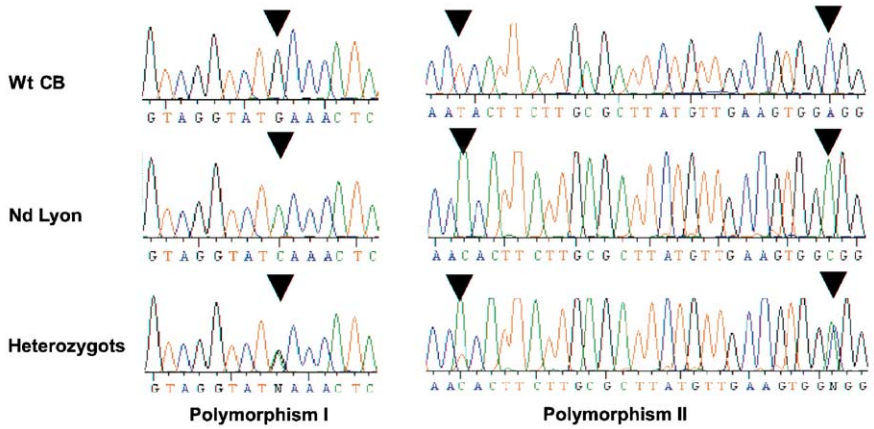

Fig. 2. Single nucleotide polymorphism in *period* DNA from wild-type strain (Wt) from C. Budejovice (CB), non-diapause mutant strain (Nd) from Lyon and their offspring. Strain specific nucleotide polymorphism is indicated by triangles.

Table 1
Results of crosses showing the mode of inheritance of the non-diapause trait in *P. apterus*

Tested females	*n*	Diapause		Reproduction	
		n	(%)	*n*	(%)
Wt	44	44	(100)	—	—
Nd	45	—	—	45	(100)
F_1 (Wt × Nd)	77	77	(100)	—	—
F_2 (Wt × Nd)	531	411	(77.4)	120	(22.6)

Diapause proportion at short-day photoperiod (12 h light/12 h darkness).

were evaluated for the diapause/non-diapause phenotype after adult ecdysis. Segregation was very close to the expected 3:1 ratio in favour of diapause females (Table 1). These results are in concert with earlier genetic analysis, including reciprocal crosses and back-crosses, suggesting that the non-diapause trait behaves as a single autosomal recessive (Socha and Hodkova, 1994).

If the non-diapause trait was linked to the *per* gene, the *per* locus of F_2 non-diapause females would be homozygous for the Lyon *per* allele. On the other hand, the *per* locus of F_2 diapause females would be either heterozygous or homozygous for C. Budejovice *per* allele. However, the results of DNA analysis did not correspond with this expectation. All *period* genotypes were associated with both diapause and non-diapause phenotypes (Table 2).

4. Discussion

We present genetic linkage analysis between the *per* gene and photoperiodism, based on single nucleotide polymorphisms in *per* DNA in geographically different strains of a heteropteran, *P. apterus*. Analysis of the *per* genotype in F_1 progeny of the wild-type strain from C. Budejovice (Czech Republic) and a non-diapause mutant strain from Lyon (France) revealed that, similar to the non-diapause trait, the *per* gene has an autosomal location. However, analysis of F_2 progeny showed that the non-diapause phenotype is not genetically linked to the *per* gene. Therefore, the function of the *per* gene in photoperiodism, if any, presumably is downstream from other, still unknown, genes controlling diapause. At present, no other information on molecular components of the circadian clock potentially linked to photoperiodism is available for a 'non-drosophilid' species. Our future long-term research will be focused on the genetic linkage analysis between photoperiodism and other clock related genes in *P. apterus*.

In a drosophilid fly, *Chymomyza costata*, non-diapause mutants show constant and low abundance of the *per* mRNA (Kostal and Shimada, 2001) and no *tim* transcripts were detected (Pavelka et al., 2003). Genetic linkage analysis demonstrated that *timeless*, but not the *per* gene, was strictly linked with the loss of the diapause response to short day-lengths in this mutant (Pavelka et al., 2003). Nevertheless, different patterns of *per* gene expression in wild-type and mutant flies deserve further attention. A potential role of *tim* gene in photoperiodism is indicated by the results on the flesh fly, *Sarcophaga crassipalpis*, showing that the amplitude of *tim* mRNA was severely damped under long day-lengths, but that of *per* mRNA was not affected (Goto and Denlinger, 2002). Furthermore, the initial clock-specific photoresponsive event in *D. melanogaster* appears to be the degradation of TIMELESS protein (Myers et al., 1996).

Non-diapause mutants of *C. costata* and *P. apterus* differ in, at least, two important characteristics: (1) Mutant females of *P. apterus* can still discriminate between short day and long day at colder temperatures

Table 2
Analysis of genetic linkage between *per* gene and photoperiodic phenotype in F_2 (Wt × Nd) females of *P. apterus*

Phenotype	Sequenced individuals (*n*)	*Period* genotype (*n*)		
		Wt C. Budejovice homozygotes	Heterozygotes	Nd Lyon homozygotes
Non-diapause	14	4	8	2
Diapause	6	2	3	1

(Hodkova and Socha, 1995), while the photoresponsiveness is completely lost in *C. costata*, irrespective of temperature (Riihimaa and Kimura, 1988). (2) Mutant females of *P. apterus* show clear circadian rhythms in locomotor activity (Hodkova et al., 2003), while the loss of photoresponsiveness in *C. costata* is associated with the loss of the adult eclosion rhythm (Kostal and Shimada, 2001).

Strong evidence against the crucial role of *per* gene in photoperiodism comes from the study on periodic mutants of *D. melanogaster*. Saunders (1990) found that flies with *per* locus defective (per^{o1}) or missing (per^{-}) were able to discriminate between long day and short day and that the circadian oscillator(s) involved in the photoperiodic time measurement have a period close to 24 h, regardless of genotype. He concluded that the *per* gene is not causally involved in photoperiodic time measurement.

Although the present findings indicate that the *per* gene is not primarily responsible for the non-diapause trait in a mutant of *P. apterus*, the magnitude of *per* gene expression may still provide an important link in the photoperiodic transmission chain. Unfortunately, this possibility cannot be directly tested because mutants in the *per* gene are not yet available in *P. apterus.* At the physiological level, *P. apterus* and *D. melanogaster* exhibit substantial differences in the photoperiodic regulation of diapause. *D. melanogaster* presents a shallow ovarian diapause, only induced at a low temperature of 12 °C (Saunders et al., 1989; Saunders and Gilbert, 1990), while *P. apterus* shows a robust diapause response at a warm temperature of 26 °C. Interactions of photoperiod and temperature in the induction of diapause were shown in a number of species, but the molecular mechanisms are not known (Saunders, 2002). Interestingly, *per* mRNA levels in heads of *D. melanogaster* are regulated by both temperature and photoperiod, with earlier accumulation and higher peak values at lower temperature and shorter photoperiod (Collins et al., 2004; Majercak et al., 1999, 2004). The authors suggest that the dual thermal and photoperiodic regulation of *per* mRNA levels acts as a sensor ensuring seasonal adaptations of activity rhythms. Whether this mechanism is also implicated in the regulation of diapause in *D. melanogaster* is not clear. A modulating effect of the *per* gene on the photoperiodic response is indicated by a considerably shorter critical day-length in periodic mutants, about 3 h in per^{o1} or 5 h in per^{-} mutants, than in a wild-type strain (Saunders, 1990). Nanda–Hamner experiments show that these effects of *per* mutations are not caused by altered circadian periods of the photoperiodic clock (Saunders, 1990, see above). Short day-lengths may lead to a lower overall temperature than long day-lengths because of differences in the duration of radiant energy absorbed by the flies. Therefore, the shorter critical day-length in periodic mutants may be due to an involvement of the *per* gene in thermal regulation of the diapause response in *D. melanogaster*. In *P. apterus*, no clear diurnal oscillation of the *per* expression were observed in heads of adults (Hodkova et al., 2003; Syrova et al., 2003). Furthermore, *P. apterus* seems to be one of the very few insects that 'measures' day-length rather than night-length, and the use of Nanda–Hamner photocycles has failed to show any obvious circadian involvement in photoperiodic time measurement (Saunders, 1987). Therefore, it is unlikely that the *per* gene has a circadian role in the photoperiodic response. Under field conditions, adults of *P. apterus* enter diapause in late summer, when temperatures are still high (Hodek, 1971). The strong up-regulation of *per* mRNA levels at short days and 26 °C (up to 10-fold, compared to long days) found in wild-type females of *P. apterus* (Hodkova et al., 2003; Syrova et al., 2003) may define a relatively high temperature threshold for the induction of diapause in this species. This raises a question of whether the lower temperature threshold for the diapause photoresponsiveness in mutant females of *P. apterus* (Hodkova and Socha, 1995) is associated with a similar decrease in the temperature threshold for the response of *per* mRNA levels to photoperiod. Future experiments are aimed at addressing this question.

Acknowledgements

We thank to Jana Mikešová for technical assistance. The study was supported by the Grant Agency of the Czech Republic (Grant 206/02/0900) and by the Grant Agency of the Academy of Sciences of the Czech Republic (Grants A5007205, Z5007907, K5052113).

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