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Evidence for a putative antennal clock in *Mamestra brassicae*: Molecular cloning and characterization of two clock genes – *period* and *cryptochrome* – in antennae

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Abstract

Circadian rhythms are generated by endogenous circadian clocks, organized in central and peripheral clocks. An antennal peripheral clock has been demonstrated to be necessary and sufficient to generate *Drosophila* olfactory rhythms in response to food odours. As moth pheromonal communication has been demonstrated to follow daily rhythms, we thus investigated the occurence of a putative antennal clock in the noctuid *Mamestra brassicae*. From moth antennae, we isolated two full-length cDNAs encoding clock genes, *period* and *cryptochrome*, which appeared to be expressed throughout the body. In the antennae, expression of both transcripts was restricted to cells that likely represent olfactory sensory neurones. Our results suggest the occurence of a putative antennal clock that could participate in the pheromonal communication rhythms observed *in vivo*.

Keywords: *Period*; *Cryptochrome*; Peripheral circadian clocks; Olfactory rhythms; *Mamestra brassicae*.

Introduction

Nocturnal insects, such as moths, mainly use olfactory cues for various aspects of their life. In particular, moths possess a highly sensitive and specific pheromonal communication, essential for sexual communication. This pheromonal communication appears as a good model for olfactory rhythms studies since it has been demonstrated to undergo daily rhythms. Indeed, the female pheromone emission, used to attract conspecific males for mating, as well as the corresponding male behavioural response, follow daily rhythms (Payne *et al*., 1970; Linn *et al*., 1996; Iglesias *et al*., 1999). However, only few studies have actually shown circadian rhythms in moth sex pheromone communication (Rosen, 2002; Rosen *et al*., 2003; Silvegren *et al*., 2005).

Circadian rhythms occur in most living organisms, from cyanobacteria to humans, and act in temporal synchronization of life processes with the environment. These rhythms are generated by endogenous circadian clocks, whose molecular basis have been shown in *Drosophila* to involve clock genes participating in transcriptional/translational-based negative feedback loops (Dunlap, 1999; Shirasu *et al*., 2003). In particular, the *period* gene (*per*) appears as a master component of the circadian clock. Its isolation and characterization were the first steps in understanding the molecular mechanisms of circadian systems in insects and vertebrates. The *per* mRNA as well as the PER protein fluctuate with a circadian rhythm and oscillations persist under constant environmental conditions, such as continuous darkness. Orthologs of *per* have been cloned in the brain of a variety of insect species, including Diptera (Colot *et al*., 1988; Nielsen *et al*., 1994; Warman *et al*., 2000), Lepidoptera (Reppert *et al*., 1994; Levine *et al*., 1995; Regier *et al*., 1998), and Hymenoptera (Shimizu *et al*., 2001). Comparative analyses of *per* genes and PER proteins in insects and mammals revealed that they could function in similar ways (Hall, 1995; Levine *et al*., 1995; Sun *et al*., 1997).

The distribution of the circadian clocks through the body and their relationships seem to be more complex than previously assumed. The view that a central master clock located in the insect central nervous system (the brain), which is necessary for behavioural rhythms such as locomotor activity, controls other rhythmic outputs has been modified in the past decade. Several lines of evidence have been accumulated for the occurence of circadian clocks in peripheral tissues. Some peripheral circadian clocks controlling non-behavioural rhythms have been discovered in insect peripheral tissues (Hall, 1995; Giebultowicz & Hege, 1997; Giebultowicz, 1999). In particular, using a *per*-driven luciferase reporter gene to monitor the circadian clock in cultured tissues from *Drosophila*, *per* has been demonstrated

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to present rhythmic expression in different tissues, including chemosensory tissues, among them the antennae (Plautz *et al*., 1997).

Another clock component, the *cryptochrome* gene (*cry*), encoding a flavoprotein belonging to the DNA photolyase blue light photoreceptor family (Todo *et al*., 1996), has been described in *Drosophila* as a circadian photoreceptor in both brain and peripheral tissues (Emery *et al*., 1998; Stanewsky *et al*., 1998; Ishikawa *et al*., 1999; Emery *et al*., 2000a; Emery *et al*., 2000b; Ivanchenko *et al*., 2001). However, it functions differentially in the central and peripheral oscillators. In the brain, *cry* is a photoreceptive molecule known to mediate light signalling to the circadian clock (Hall, 2000). In peripheral tissues, *cry* has been shown to be not only a photoreceptive molecule but also an integral component of the endogenous clock mechanism, in particular in *Drosophila* antennae (Ivanchenko *et al*., 2001; Krishnan *et al*., 2001).

Rhythmic olfactory responses have been described in Diptera and Blattaria insect orders (Krishnan *et al*., 1999; Page & Koelling, 2003), by electroantennography (EAG), which measures the electrical response of antennae after odour-induction (Krishnan *et al*., 2005). In *Drosophila*, olfactory rhythms are abolished in *per* null-mutants as well as in transgenic lines in which *per* expression is restricted to optic lobe's neurones (Krishnan *et al*., 1999), suggesting that peripheral circadian oscillators are necessary for circadian rhythms in olfactory responses. In a similar way, a loss of EAG rhythmicity has been observed in *Drosophila cry*^{b} loss-of-function mutant, whose central master clock remains functional. This suggests that the corresponding CRY protein could contribute to oscillator function in the antennae (Krishnan *et al*., 2001). Moreover, using targeted ablation of lateral neurones and clock disruption in antennal neurones, Tanoue *et al*. (2004) demonstrated that circadian clocks in antennal neurones are not only necessary but sufficient for olfactory rhythms in *Drosophila*. On the contrary, olfactory rhythms, measured as EAG in the cockroach *Leucophaea maderae*, have been demonstrated to be driven by the optic lobes, known to regulate the locomotor activity. By contrast, no EAG rhythm could be observed in the antennae of the Lepidoptera *Agrotis segetum* under pheromonal stimulation, although the male behavioural responses were demonstrated to be under the control of an endogenous clock (Rosen *et al*., 2003). Could Lepidoptera then use different mechanisms than Diptera and Blattaria for the control of their olfactory rhythms?

To investigate this question, we focused our work on the possible occurence of an antennal peripheral clock in the moth *Mamestra brassicae* to further investigate its contribution to the observed olfactory communication rhythms in Lepidoptera. The two circadian clock genes, *per* and *cry*, were identified and characterized in the antennae as they were demonstrated to contribute to antennal oscillator function in *Drosophila*. Their expression patterns were studied in the different parts of the body with a special emphasis on the olfactory organs, the antennae. In particular, this led us to establish for the first time *cry* expression pattern in this tissue.

Results and discussion

Gene isolation

Two clock genes, *per* and *cry*, were isolated from *M. brassicae* antennae.

Using PCR and RACE-PCR strategies, we isolated an antennal cDNA sequence that showed conservation with known *per* genes from other species, and thus that was called *Mbra-per* (GenBank accession number: AY485221). Several clones were sequenced and showed 100% identity, suggesting that the amplified PCR product could correspond to a single transcript. *Mbra-per* was 3017 bp-long and contained an open reading frame (ORF) encoding a putative 778 amino acid protein, Mbra-PER, with a calculated molecular weight of 87.16 kDa. Alignment of Mbra-PER with PER proteins from other insects (Fig. 1A,B) revealed significant amino acid similarities. Mbra-PER was closer to PER from other lepidopteran sequences: it presented 59 and 51% of identities with the sequences of *Bombyx mori* and *Antheraea pernyi*, respectively. Compared to other insect orders, it presented only 29% of identity with the most abundant PER isoform (PER A) of *Drosophila melanogaster*, demonstrated to be implicated in clock function, and only 27 and 26% of identity with, respectively, the Blattaria *Blatella germanica* and *Periplaneta americana* PER sequences. Sequences comparison revealed that Mbra-PER presented several conserved domains, named C1 to C3 by Colot *et al*. (1988) (Fig. 1A). In particular, various motifs important in the clock mechanism were well conserved: the PAS (Per-Arnt-Sim) domain and the CLD (Cytoplasmic Localization Domain). PAS, located in C2 (Fig. 1A,B), is an heterodimerization domain characteristic of a family of transcription factors, known to mediate interactions between PER and other clock proteins (Dunlap, 1999). CLD domain regulates nuclear entry of the PER/TIMELESS heterodimer, a critical step in the *Drosophila* clock mechanism (Saez & Young, 1996). The CLOCK/CYCLE inhibition domain (CCID), that contains the C3 region (Fig. 1A), was less conserved, although it is functionally crucial for the repressive activity of PER on the CLOCK/CYCLE heterodimer in *Drosophila* by inhibition of CLOCK/CYCLE-mediated transcription (Chang & Reppert, 2003). Conservation was also low in the C-terminal end of the protein, as already observed among known PER proteins, suggesting that this part of the protein may not be critical in the clock mechanism. As the function of this part of the protein is not yet well understood, it has been alternatively proposed that it might be involved in the temporal regulation of

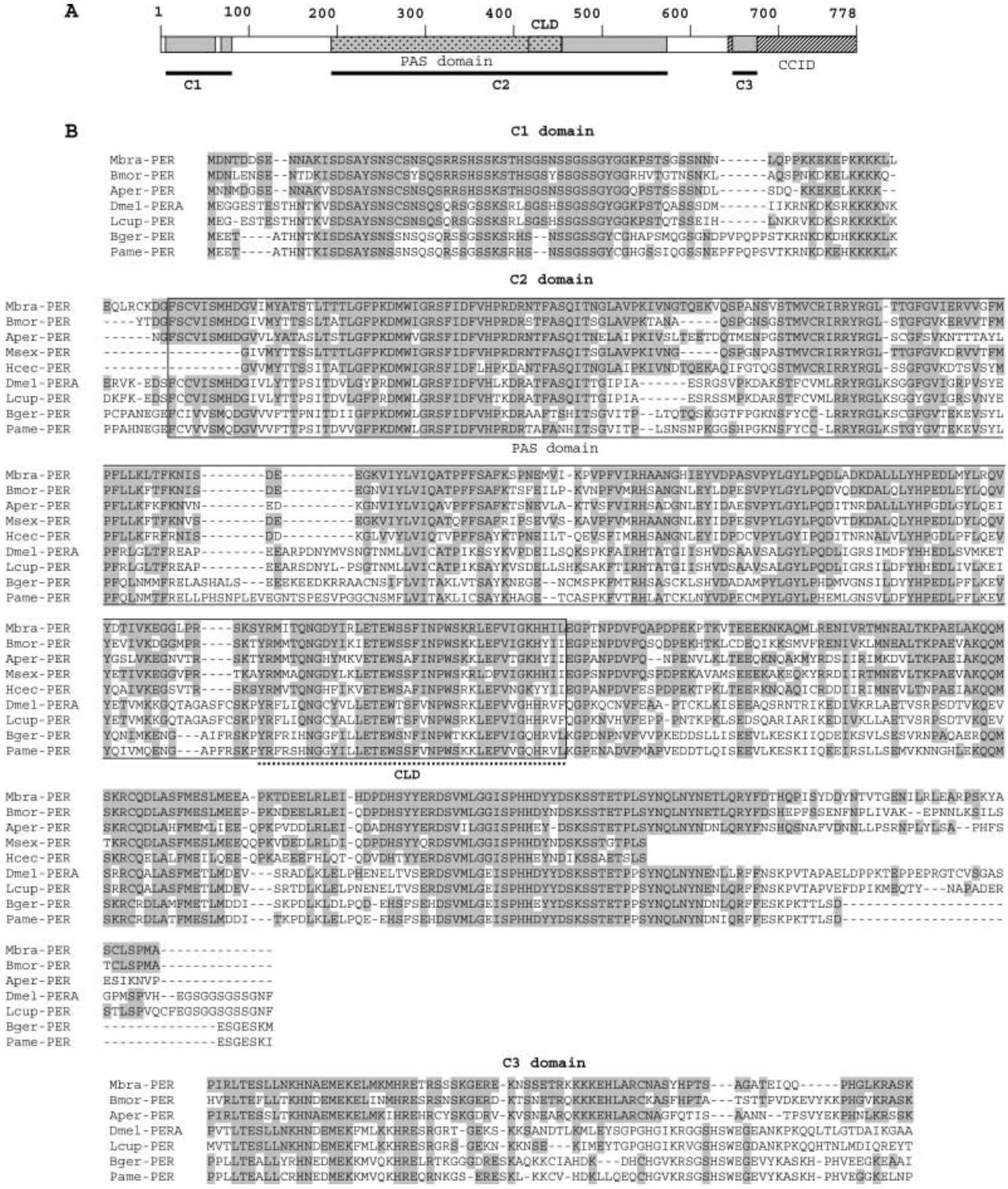

Figure 1. (A) Schematic representation of the predicted amino acid sequence of Mbra-PER. The boxes in grey represent the three conserved regions (C1 to C3) as defined (Colot *et al*., 1988). Into the C2 region, the dotted box represents the Per-Arnt-Sim (PAS) domain including the Cytoplasmic Localization Domain (CLD). At the C-terminal end, the hatched box represents the CLOCK/CYCLE inhibition domain (CCID) containing the C3 region. The numbers on the top correspond to the size in amino acids. (B) Alignment of the conserved domains C1 to C3 of the deduced protein sequence of *Mbra-per* with PER from other insect species. Mbra-PER: *Mamestra brassicae* period (GenBank accession number AY485221); Bmor: *Bombyx mori* (AY526605); Aper: *Antheraea pernyi* (U12769); Msex: *Manduca sexta* (U12773); Hcec: *Hyalophora cecropia* (U12771); Dmel-PERA: *Drosophila melanogaster* type A PER (M30114); Lcup: *Lucilia cuprina* (Y19108); Bger: *Blatella germanica* (AF297552); Pame, *Periplaneta americana* (U12772). The amino acids highlighted in grey are conserved between *M. brassicae* and other species. The PAS domain of C2 region is boxed and the CLD domain is dotted underlined.

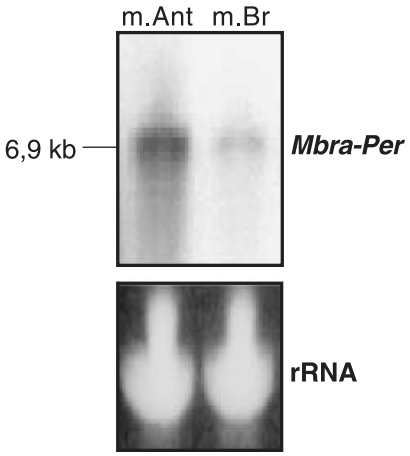

Figure 2. Northern blot analyses of *Mbra-per* transcripts in *Mamestra brassicae* male brains (m.Br) and antennae (m.Ant), revealed by chemiluminescent detection. RNA loading was controlled by ethidium bromide staining of rRNA (bottom).

behaviours characteristic of each insect group (Shimizu *et al*., 2001).

Since two *per* transcripts have been shown to occur in different species, such as *D. melanogaster* (Cheng *et al*., 1998), we performed Northern blot with RNA from male brains and antennae in order to get an estimation of the number and size of transcript(s). A clear and unique hybridization band was observed for each tissue tested (Fig. 2). However, the size of the transcript, estimated around 7 kb, was larger than the size of the cDNA we obtained (2,3 kb), suggesting that this transcript might carry a large amount of non-coding mRNA, not fully amplified with the 3′ RACE procedure. This phenomenon has been already observed in other non-dipteran species, such as the Lepidoptera *A. pernyi* and the cockroach *P. americana*, in which *per* cDNAs were, respectively, 2,7 and 3,6 kb-long, whereas the sizes of the corresponding transcripts were 8 kb and 9,5 kb (Reppert *et al*., 1994).

We also report the characterization in male antennae, of another gene showing conservation with known *cry* genes, therefore called *Mbra-cry* (GenBank accession number: AY947639). The full-length sequence of 1834 bp contained an ORF encoding a 548 amino acid polypeptide, Mbra-CRY, with a putative molecular weight of 62,9 kDa. Alignment of the antennal Mbra-CRY amino acid sequence with CRYs from other insect species showed high sequence similarities (Fig. 3). Mbra-CRY was very close to *A. pernyi* CRY (86% identity), the unique lepidopteran ortholog known to date, whereas it shared less than 50% with CRY from the Diptera *D. melanogaster* and *Bactrocerca tryoni*. In particular, Mbra-CRY presented two characteristic conserved domains: the DNA photolyase and the Flavin-Adenine Dinucleotide (FAD) binding domains (Fig. 3). These two domains form the CRY's photolyase homology domain, which has recently been demonstrated to be sufficient for light detection and phototransduction in *Drosophila* (Busza *et al*. 2004). The structural conservation of the DNA photolyase and the FAD binding domains between the different CRY proteins could suggest common mechanistic features, particularly in photoreception. Moreover, inside these domains, amino acids putatively involved in binding with 5, 10-methenyltetrahydrofolate (MTHF) and FAD cofactors, and cyclobutane pyrimidine dimer (CPD) (Emery *et al*., 1998), were also conserved (Fig. 3).

We then have identified in *M. brassicae* male antennae two new *per* and *cry* ortholog genes. Interestingly, when comparing the sequence identities within PER and CRY proteins between the same species, *M. brassicae*, *A. pernyi* and *D. melanogaster*, it appears that PER sequences are more divergent than the CRY ones. This observation corroborates the fact that CRY has been more conserved than PER during evolution (An *et al*., 2004).

Mbra-per *and* Mbra-cry *expression*

The tissue-related expressions of *Mbra-per* and *Mbra-cry* were determined by RT-PCR on various tissues from adult males and females. In addition to the brain, which is known to support the cerebral clock, peripheral tissues were used, including male and female antennae as well as male proboscis, legs, thorax and abdomens. The ubiquitous ribosomal gene *rpL8* was used as a positive control (Fig. 4). For similar levels of *rpL8* expression, both transcripts were amplified in all tissues tested, with a very faint amplification observed in male abdomens for *Mbra-per*. Such a *per* and *cry* expression throughout the body has already been observed in *Drosophila* (Plautz *et al*., 1997; Okano *et al*., 1999). Interestingly, *Mbra-per* and *Mbra-cry* were expressed in chemosensory tissues: proboscis and legs, where taste sensilla are located, and olfactory organs, such as male and female antennae. Taken together, our results suggested that peripheral clocks could occur in the different parts of *M. brassicae* body, and in particular, clear expression of both genes in the antennae suggested the existence of an antennal clock. However, in absence of circadian expression studies, one can not exclude a possible non-clock function of these genes in peripheral tissues. Indeed, two *Drosophila* clock genes, *period* and *timeless*, have been shown to present non-circadian expression in the ovaries, suggesting that they could be involved in a novel non-circadian function in this organ (Beaver *et al*., 2003).

Mbra-per *and* Mbra-cry *expression in olfactory sensory neurones*

More information came from the expression pattern studies of *Mbra-per* and *Mbra-cry* in *M. brassicae* male antennae, using *in situ* hybridization (Fig. 5). *M. brassicae* antennae are filiform, approximately 1 cm long and comprise about 72 segments. Each segment exhibits the same general

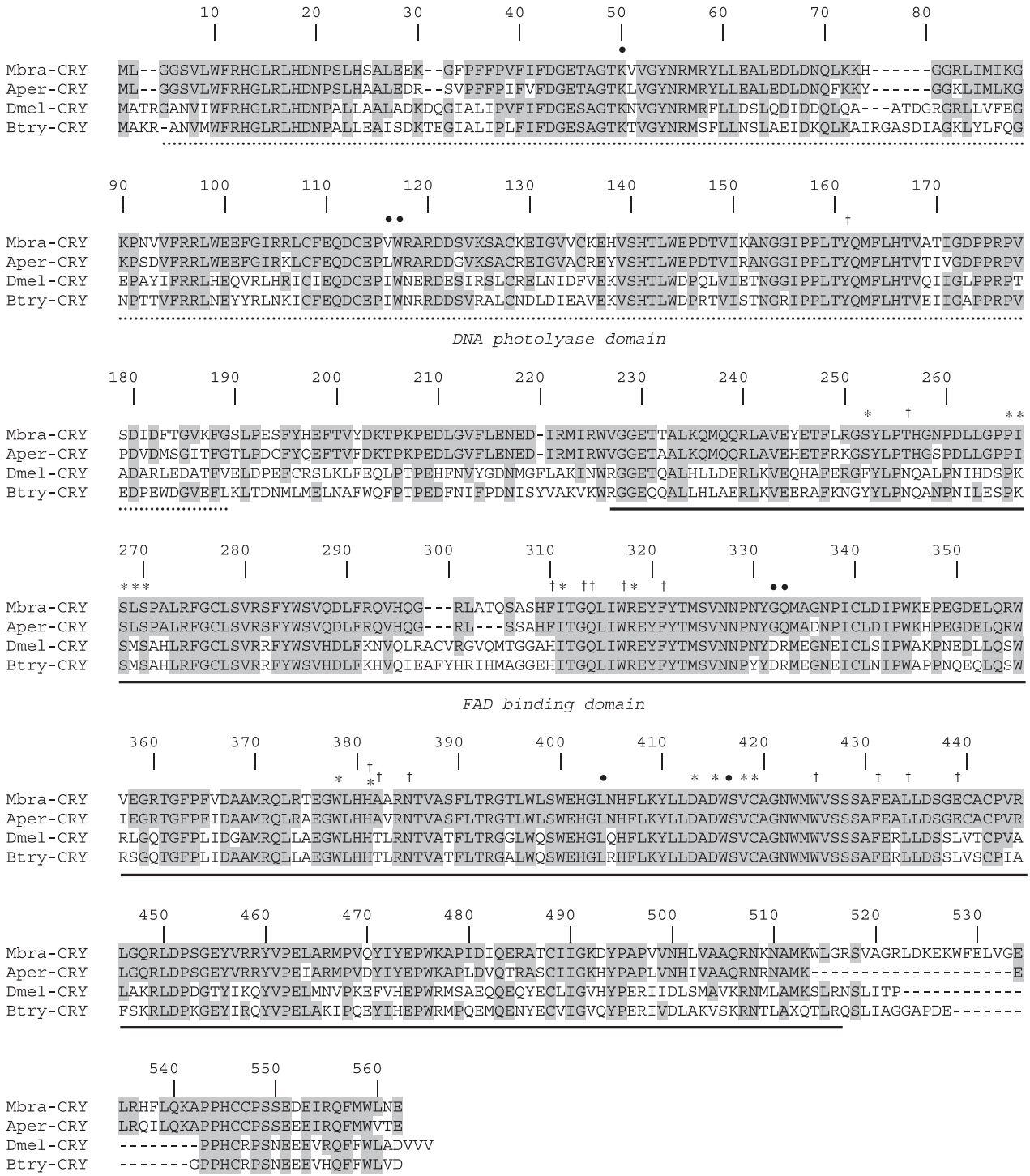

Figure 3. Alignment of the predicted amino acid sequence of Mbra-CRY (GenBank accession number AY947639) with CRY proteins from other insect species, including *Antheraea pernyi* (Aper) (AF333998); *Drosophila melanogaster* (Dmel) (AF099734); *Bactrocerca tryoni* (Btry) (AY708049). The amino acids highlighted in grey are conserved between *Mamestra brassicae* and the other species ●: 5, 10-methenyltetrahydrofolate (MTHF) binding amino acids; *: Flavin-Adenine Dinucleotide (FAD) binding amino acids; †: cyclobutane pyrimidine dimer (CPD) binding amino acids, as defined (Emery *et al*., 1998). The DNA photolyase domain is perforated underlined and the FAD binding domain is underlined.

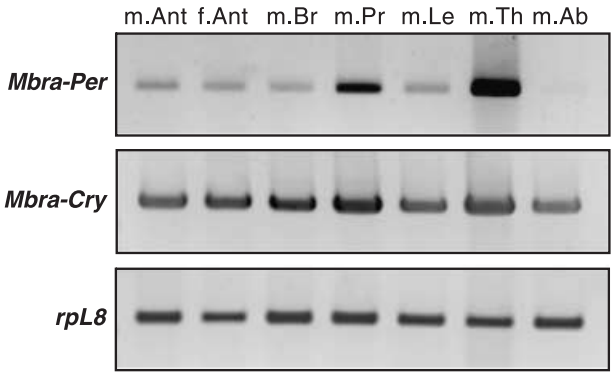

Figure 4. RT-PCR study showing expression of *Mbra-per, Mbra-cry* and *rpL8* (control) in various tissues of *Mamestra brassicae* adults, including male antennae (Ant), brains (Br), proboscis (Pr), legs (Le), thorax (Th), abdomens (Ab) and female antennae (Ant). m: male; f: female.

organization: the dorsal side is covered with two rows of scales and the olfactory hairs, the sensilla trichodea, are located on the ventral side. In males, the olfactory hairs belong to two morphological classes according to their length. The long sensilla trichodea (60–190 μm) are located on the lateral parts of the ventral area and are ranged in four to five parallel rows. The short sensilla trichodea (35–55 μm) are located medio-ventrally and are not arranged in rows (Renou & Lucas, 1994). The two *Mbra-per* and *Mbra-cry* genes appeared to share the same expression pattern in the antennae. Whereas sense strand controls gave no signal (not shown), antisense probe hybridizations were restricted to the ventral side of the antennae that carries the olfactory sensilla (Fig. 5 A,D), with no labelling on the scale side. Closer examination revealed that signals were located just below the cuticle, in cells at the base of both long and short olfactory hairs (Fig. 5B,C,E,F,G). Olfactory sensilla house one or several bipolar olfactory neurones that are surrounded by 3 accessory cells. It was difficult to conclude about the exact nature of the *Mbra-per* and *Mbra-cry* labelled cells (neurones or accessory cells), but careful examination revealed some labelling inside the sensilla that may correspond to the dendrite of the neurone (Fig. 5B,G; arrows). Furthermore, two labelled somata could be seen at the base of some sensilla in antennae hybridized with *Mbra-per* probe (Fig. 5C; arrows). They could represent the two olfactory neurones of the same sensilla. Although *per* labelling has already been observed in *Drosophila* antennae at the base of the sensilla (Plautz *et al*., 1997), in this study, we suggest a specific *per* expression in moth olfactory neurones. To our knowledge, *cry* expression pattern in insect antennae has never been investigated to date. In this study, we reported for the first time its precise expression pattern in the antennae of a lepidopteran species. Our results argue that *cry* could also be expressed in olfactory neurones. For the two genes *Mbra-per* and *Mbra-cry*, labelled spots were associated with the two sensilla types, the short and long ones, and labelling seemed to be associated with most, if not all, the olfactory sensilla. Thus, both genes could be proposed to be coexpressed in the same cells. As these two genes represent key components of circadian clocks, their expression in antennae suggested the occurence of a peripheral antennal clock in *M. brassicae*, located in sensory neurones of olfactory structures. Moreover, the presence of *cry* in the antennae suggested that this putative antennal clock could be directly light-sensitive.

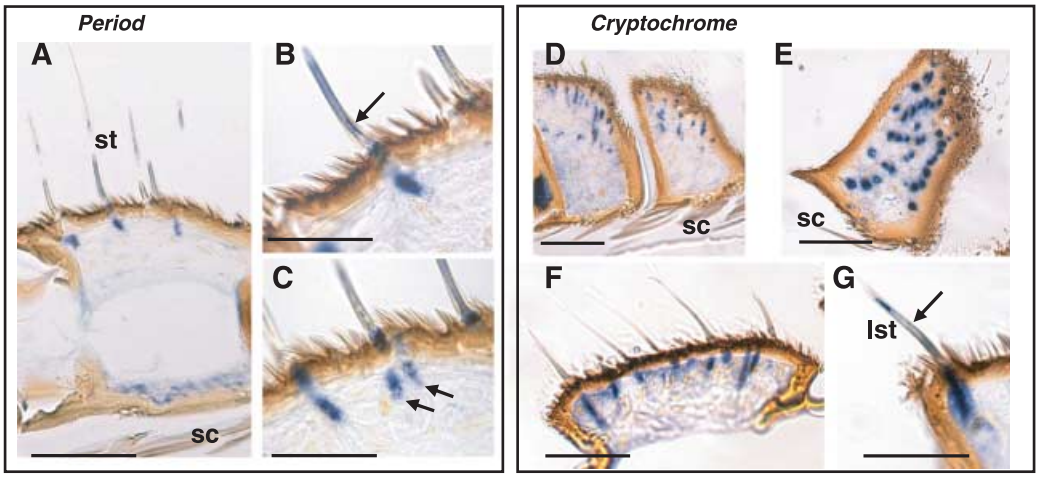

Figure 5. Expression pattern of *Mbra-per* (A-C) and *Mbra-cry* (D-G) revealed by *in situ* hybridization on longitudinal sections of male antennae. (A) *Per* expression on the sensilla side of the antennae with labelled cells located at the base of olfactory sensilla trichodea (st); sc: scales. (B, C) Sensilla trichodea at higher magnification. (B) Labeling inside a sensillum (arrow). (C) Two labelled somata at the base of a sensillum trichodeum (arrows). (D) *Cry* expression on the sensilla side of the antennae. (E) Section through the cuticle, showing labelling arranged in parallel rows, following the typical disposition of long sensilla trichodea. (F) Complete antennal segment section, showing the labelling at the base of the sensory hairs. (G) Detail of a long sensillum trichodeum, with labelling into the sensilla. Bars: 50 μm in (A, D, E, and F), 20 μm in (B, C, and G).

Although no data are available on *per* expression pattern in *M. brassicae* brain neurones, PER has been demonstrated to be expressed in a variable number of brain cells according to the lepidopteran species. Whereas the brains of both silkmoths *A. pernyi* and *B. mori* contain only a few putative circadian clock cells (Sauman & Reppert, 1996; Sehadova *et al*., 2004), a widespread *per* expression has been found in *Manduca sexta* neural tissues (Wise *et al*., 2002), although most neurones did not appear to be rhythmic. *M. brassicae* antennae carry a large number of olfactory sensilla containing olfactory neurones, whose number can be estimated around 15 000 neurones per antennae (Renou, pers. comm, INRA, Versailles). The difference in the *per* expression level between brains and antennae observed in the Northern blot (Fig. 2), in which equal quantity of RNAs were loaded, could thus reflect a low number of brain cells expressing *per* compared to antennal cells.

In this paper, we have characterized two clock genes, *Mbra-per* and *Mbra-cry*, isolated from male antennae. Their tissue distributions, studied by RT-PCR, revealed an ubiquitous expression of both genes throughout the body, including the antennae. In these olfactory organs, *Mbra-per* and *Mbra-cry* expressions were to be restricted to cells that are likely appear as olfactory neurones, and both genes appeared to be co-expressed in the same cells. Taken together, our results provide strong evidence for the occurence of a putative antennal clock located in olfactory sensilla in the moth *M. brassicae*. However, the function of these two clock genes in antennae is still unknown since the temporal variations of *per* and *cry* expressions have not been studied yet in *M. brassicae* antennae.

Experimental procedures

Animals and tissue collection

Animals were reared in Domaine du Magneraud (INRA, France) and sexed as pupae. Pupae were maintained under light-dark (LD) 16 : 8 cycles at 24 °C and 70% humidity until emergence. For cDNA synthesis, tissues were dissected from sexually mature 3-day-old adults (antennae, proboscis, brains, legs, wings, thorax, and abdomens) and stored at –80 °C until use. For *in situ* hybridization, antennae were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, then dehydrated in methanol, and stored at –20 °C until use.

RNA extraction and cDNA synthesis

Total RNAs were extracted from the different tissues with TRIzol® reagent (Invitrogen, The Netherlands) and quantified by spectrophotometry at 260 nm. Single stranded cDNAs were synthesized from 1 μg of total RNAs with M-MLV reverse transcriptase (Clontech, USA) using buffer and protocol supplied in the Advantage™. RT-for-PCR Kit (Clontech). For the 3′ and 5′ RACE, cDNAs were synthesized from 1 μg of male antennae total RNAs at 42 °C for 1.5 h using the SMART™ RACE cDNA Amplification Kit (Clontech) with 200 U of Superscript II (Gibco, BRL), 3′ CDS-primer and SMART II oligonucleotide, according to the manufacturer's instructions.

Molecular cloning of M. brassicae per *ortholog cDNA in antennae*

Antennal cDNA was used in PCR with two degenerate primers, *PerUp* (5′GCAGGTCGTTYATYGAYTTCSTTCA-3′) and *PerDown* (5′-TCCTGGGGDAGATAKCCVAGRTACGG-3′), designed in the C2 conserved region of PER proteins from different lepidopteran species, *A. pernyi* (GenBank accession number: U12769), *Hyalophora cecropia* (U12771) and *M. sexta* (U12773): GRSFIDFVH and PYLGYLPQD, respectively. PCR consisted of 40 cycles at 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min. The generated 429 bp fragment was gel purified (GenElute™, USA, Sigma) and cloned into pCR® II-TOPO® plasmid (Invitrogen). After transformation of competent bacteria, the positive clones were subcultured and the recombinant plasmids were isolated by mini preparation (QIAprep Spin Miniprep Kit, Qiagen, France). Recombinant plasmids were digested with *Eco*RI enzyme to screen for the presence of insert and then sequenced (Genome Express, France). Gene sequence analyses and database comparisons were performed using the Blast program (Altschul *et al*., 1990), MWCALC (@Infobiogen, Eury, France), GENEJOCKEY II software (Biosoft, Cambridge, UK) and alignment were carried out using ClustalW (NPS@IBCP, Lyon, France). Several clones were sequenced and presented 100% identity.

The 3′ and 5′ regions of the corresponding cDNA were obtained after 3′and 5′ RACE-PCRs, according to the SMART™ RACE kit instructions (Clontech) using Universal Primer Mix vs. gene-specific primers: 5′-CGCTACAGAGGCCTTACAACCGGCTTCGG-3′ for the 5′ RACE and 5′-CCGAAGCCGGTTGTAAGGCCTCTGTAGCG-3′ for the 3′ RACE. Touchdown PCRs were performed as follows: after 1 min at 94 °C, five cycles of 30 s at 94 °C, and 3 min at 72 °C, then five cycles of 30 s at 94 °C, 30 s at 70 °C, and 3 min at 72 °C, then 30 cycles of 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C, then 10 min of final elongation step at 72 °C. The PCR products were cloned, sequenced, and analysed as described above. By merging the overlapping sequences obtained by 3′ and 5′ RACE, a cDNA of 3017 bp, called *Mbra-per*, was generated and identified as a putative *period* ortholog after sequence analyses.

Molecular cloning of M. brassicae *cry ortholog cDNA in male antennae*

Two degenerate primers were designed according to conserved regions of *cryptochrome* sequences from different insect species, including the Lepidoptera *A. pernyi* (GenBank accession number AF333998) and the two Diptera, *D. melanogaster* (AF099734) and *Sarcophaga crassipalpis* (AB079536). The nucleotidic sequence of the sense primer *CryUp* was based on the amino acid motif FDGEXAGTK (5′-TTCGATGGAGAGASTGCAGGTACYAA-3′) and the antisense primer sequence, *CryDown*, was based on the amino acid sequence AGNWMWV (5′-ACCCACATCCARTTICC-3′). PCR consisted of 40 amplification cycles with annealing temperature of 50 °C and generated a 1125-bp product. This product was cloned, sequenced, and analysed as described above.

Amplifications of the 5′-and 3′ regions of the cDNA were obtained by 5′-and 3′ RACE PCRs with a specific antisense primer (5′-GGACGCGGAGGATCGCCGATAGTCGC-3′) and a specific sense primer (5′-GCCAACGGCGGCATCCCTCCTCTT-3′), used in touchdown PCRs performed as described above, excepting a final annealing temperature of 65 °C. PCR products were purified, cloned and sequenced as described. By merging the overlapping sequences obtained with the internal fragment, a putative full-length cDNA of 1780 bp was generated, designed as *Mbra-cry*.

Northern blot of per *in male brains and antennae*

A specific digoxigenin labelled probe was produced by PCR on antennal cDNA using the Dig-DNA labelling mix (Roche, Roche Diagnostics, France) and two *Mbra-per* specific primers (5′-GGCAGGTCGTTYATYGAYTTCSTTCA-3′ and 5′-TCCTGGGGD-AGATAKCCVAGRTACGG-3′). Total RNAs (20 μg) from male brains and antennae were separated on a 1% formaldehyde gel and transferred on to a positively charged nylon membrane. Equal loading was monitored by comparing the density of the ribosomal RNA band under UV on the agarose gel and membrane, before and after transfert. Overnight hybridization (45 °C), washes and chemiluminescent detection were performed as described previously (Maïbèche-Coisné *et al*., 2002).

Tissue expression of Mbra-per *and* Mbra-cry *by RT-PCR detection in different tissues*

Equal amounts of RNAs (1 μg) extracted from male antennae, brains, proboscis, legs, thorax, and abdomens, and from female antennae were treated with DNase1 (Promega, France) and reverse-transcribed (Advantage™ RT-for-PCR kit; Clontech). *Mbra-cry* cDNA was amplified with the following couple of primers: 5′-GCCAACG-GCGGCATCCCTCCTCTT-3′ and 5′-GCCGGTGCGGCCTTC-GACCCATCT-3′, generating a 621 bp fragment. *Mbra-Per* cDNA was amplified with: 5′-TACAGAGGCCTTACAACCGG-3′ and 5′-AGAGCCTCATTCATGGTCCG-3′, to obtain a fragment of 620 bp. *RpL8,* encoding a *M. brassicae* ribosomal protein, was used as a 508 bp control, as already described (Maïbèche-Coisné *et al*., 2004). Amplification products were loaded on 2% agarose gels and visualized with ethidium bromide.

In situ *hybridization*

Dig-labelled RNA sense and antisense probes (430 and 306 bp-long for *per* and *cry*, respectively) were *in vitro* transcribed from PCR fragments amplified from the recombinant plasmids *per*-pCR®II-TOPO® and *cry*-pCR®II-TOPO® with M13 Forward and M13 Reverse primers. The transcriptions were performed using T7 and SP6 RNA polymerases (Promega) following recommended protocol to generate both *per* and *cry* sense and antisense probes. The probes were purified with RNA G50 sephadex columns (Quick Spin columns, Roche).

The hybridization protocol was performed on whole-mount pieces of antennae, as described (Jacquin-Joly *et al*., 2000). Longitudinal sections were performed at 6 μm and counter-stained with acridine orange. Sections were photographed, and then pictures were digitized and processed using Adobe Photoshop 7.0 (USA).

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