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Short Communications

Analysis of the expression pattern of the defensin gene in the lepidopteran *Mamestra brassicae*

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Abstract

Southern blotting experiments performed on *Mamestra brassicae* genomic DNA after digestion with methylation-sensitive restriction enzymes indicated that defensin gene is methylated at CpG targets in the promoter region. However, defensin gene is actively transcribed despite the presence of methylation. Experiments performed by genome demethylation indicated that demethylated defensin gene resulted in an altered expression after bacterial induction. In particular, no increase in gene expression was observed after induction with Gram positive bacteria if defensin gene was demethylated. The present results are intriguing since they indicate that in *M. brassicae* DNA methylation is not involved in gene silencing, but also that DNA methylation could be essential to assure the expression of specific genes. On the whole, data here presented argue against an unifying and evolutionary conserved role of cytosine methylation from invertebrates to vertebrates.

Key words: epigenetics; DNA methylation; defensin gene expression; lepidopteran

Introduction

Defensins are 4kDa cationic peptides with a characteristic six cysteine/three disulfide bridge pattern and three domains consisting in a flexible amino-terminal loop, a central α-helix and a carboxy-terminal antiparallel β-sheet (Hoffmann and Hetru, 1992; Hetru *et al*., 1998; Bulet *et al*., 1999).

Several authors reported the isolation and description of defensins or defensin-like peptides in different insects (Chalk *et al*., 1995; Lowenberger *et al*., 1995; Cho *et al*., 1996, 1997; Miyanoshita *et al*., 1996; Richman *et al*., 1996; Dimopoulos *et al*., 1997; Gao *et al*., 1999; Muller *et al*., 1999; Eggleston *et al*., 2000), but just two papers reported the presence of defensin-like peptides in Lepidoptera (Lamberty *et al*., 1999; Mandrioli *et al*., 2003). In particular, Lamberty and colleagues identified in *Heliothis virescens* a peptide, named heliomicin, that shows antifungal activity and shares similarities with antibacterial insect defensins (Lamberty *et al*., 1999). Mandrioli and colleagues (2003) isolated a defensin gene that results highly conserved at a sequence level. It has also been indicated that, even if a weak constitutive expression of the defensin gene can be detected, defensin gene expression was greatly increased by Gram positive, but not by Gram negative bacteria (Mandrioli *et al*., 2003).

The present paper analyses the methylation status of the defensin gene and the functional significance of methylation for defensin gene expression in the immunocytes of the cabbage moth *Mamestra brassicae*.

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Materials and Methods

Samples

The IZD-MB-0503 immunocyte cell line from the insect *Mamestra brassicae* (Lepidoptera) (ATCC number: CRL-8003) was used. The cells were cultured in Ex-Cell 405 medium (JRH Biosciences, KS, USA) at 26 °C.

Presence of defensin molecules

The presence of defensin molecules was detected in the IZD-MB-0503 cell line by immunocytochemical procedure and *in situ* hybridisation according to Mandrioli *et al*. (2003).

PCR amplification of defensin probe, genome demethylation and Southern blotting

Genomic DNA extraction from the IZD-MB-0503 cell line, electrophoresis and transfer of the DNA from agarose gel to nylon membrane were performed following Mandrioli (2002). PCR amplification of a portion of the defensin gene was carried out using two primers: F (5'-CCAAATGCCTCGTCATCT) and R (5'-ATTAGAGTC AAGCTCAAAAGGG). Primers were selected according to *M. brassicae* defensin gene sequence (AF465486) available in GenBank (Mandrioli *et al*., 2003). The amplification mix contained 100 ng of genomic DNA, 1 μM of each primer, 200 μM dNTPs and 2U of DyNAZyme II polymerase (Finnzymes Oy, Finland). Amplification was performed with a thermocycler at an annealing temperature of 55 °C for 30 sec and extension at 72 °C for 45 sec. The amplified portion of the defensin gene was labelled using the Dig High Prime kit (Roche) according to manufacturer's instructions.

Genome demethylation was obtained by treating *in vitro M. brassicae* cells with a 20 mM 5-azacytidine solution for 72 hours according to Mandrioli and Volpi (2003). Southern blotting has been performed according to Mandrioli (2002).

Analysis of defensin gene expression by Northern blotting and RT-PCR

RNA extraction was carried out using the "SV Total RNA Isolation kit" (Promega) following the manufacturer's protocols. RNA was successively electrophoresed on formaldehyde gel, transferred onto a nylon membrane by capillary transfer and blotted with the digoxygenin (DIG)-labelled defensin probe following standard techniques. Northern blotting was carried out following Mandrioli (2002). RT-PCR amplification was performed according to Ottaviani *et al*. (2002).

Results and Discussion

Southern blotting performed on *M. brassicae* genomic DNA after digestion with methylation-sensitive restriction enzymes (*Msp*I, *Hpa*II, *Mbo*I and *Sau*3AI) clearly indicated that the defensin gene is methylated (Fig. 1a-b). In particular, comparison of the restriction pattern of *Msp*I and *Hpa*II showed that methylation is confined to CpG targets. *Msp*I and *Hpa*II are, in fact, isoschizomers that recognize the same target sequence 5'-CCGG-3', but while *Msp*I cleaves its target irrespectively of the methylation of the inner cytosine of the target sequence, *Hpa*II is inhibited by its methylation. The distribution of the *Msp*I restriction targets inside the defensin gene sequence, together with the results of the Southern blotting experiments, suggested that methylation could be limited to the 5' promoter region of the gene.

In order to verify the effect of methylation on gene expression we analyzed the presence of both defensin mRNA and peptide. The presence of defensin transcripts has been verified using RT-PCR, and *in situ* hybridisation (Figs 1, Fig. 2a), whereas gene expression was assessed using *in situ* immuno-procedures (Fig. 2b). All the applied techniques clearly indicated that defensin gene is constitutively expressed despite the presence of methylation at the promoter region of the gene.

In order to verify if gene demethylation was related to alteration in defensin expression, *M. brassicae* genome was demethylated by treating cabbage moth cells with a 20 mM 5-aza-cytidine solution for 72 h. The relationship between level of methylation and degree of defensin gene expression has been evaluated both at a constitutive level and after induction with the Gram positive bacteria *Staphylococcus aureus* (ATCC 6538).

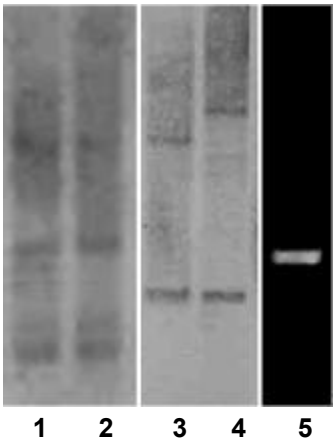

Fig. 1 Southern blotting with the defensin probe on cabbage moth DNA digested with *Mbo*I (lane 1), *Sau*3AI (lane 2), *Msp*I (lane 3) and *Hpa*II (lane 4) evidenced the presence of methylated CpG upstream the defensin gene. RT-PCR experiments indicated that defensin gene is expressed despite gene methylation.

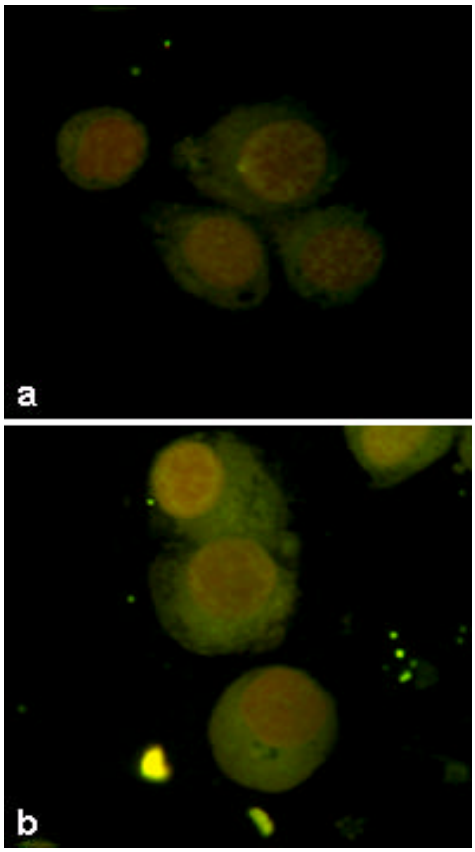

Fig. 2. Experiments of *in situ* hybridisation (a) and immuno-cytochemistry (b) indicated that defensin gene is constitutively expressed.

RT-PCR and northern blotting experiments showed that the constitutive expression of the defensin gene was not altered by genome demethylation (Fig. 3). On the contrary, genome demethylation completely abolish gene up-regulation after induction by Gram positive bacteria. The present findings demonstrated that methylation of the cabbage moth defensin gene is not important for the constitutive expression of the gene, but is essential for its up-regulation after bacterial challenge (Fig. 3). These results are very intriguing since they indicate not only that in *M. brassicae* DNA methylation is not involved in gene silencing, but also that cytosine methylation could be essential to assure the expression of specific genes. These findings are in agreement with those reported in other insects, such as *Myzus persicae* (Hick *et al*., 1986; Field *et al*., 1989; Field, 2000) and *Planococcus citri* (Bongiorni *et al*., 1999; Bongiorni and Prantera, 2003), where methylated genes resulted to be highly expressed and DNA methylation was fundamental to assure their expression. The above reported data argue against an unifying and evolutionary conserved role of cytosine methylation from invertebrates to vertebrates. In fact, it appears that the DNA methylation/gene silencing correlation, which is typically reported in vertebrates (Wolffe *et al*., 1999), is not true for insects. Methylation in insects could be

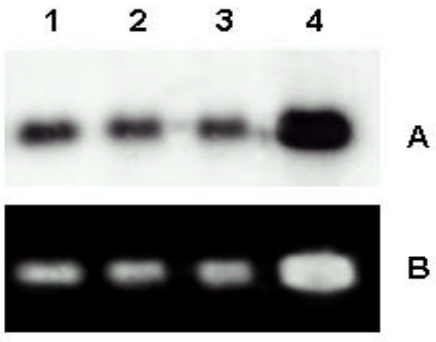

Fig. 3. Northern blotting experiments (A) and RT-PCR amplifications performed using RNA samples extracted from non-induced control cells (lane 1), demethylated non-induced cells (lane 2), demethylated induced cells (lane 3) and induced control cells (lane 4).

essential to focus initiation of transcription on genuine promoters in order to guarantee the expression of specific genes susceptible to transcriptional interference. In view of this assumption, insects methylate genes that have to be expressed in place of silent ones.

In conclusion, we hypothesize the presence of a discontinuity in the functional role of methylation from invertebrates to vertebrates since it appears that the DNA methylation/gene silencing correlation is not present in insects.

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