RESEARCH REPORT

Molecular and cellular analysis of immunity in the phytoplasma vector *Euscelidius variegatus*: exploiting immunity to improve biological control strategies

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Abstract

Insects depend on innate immunity only to defend themselves against pathogens and to regulate interactions with many other microorganisms, such as different kinds of symbionts. Recently, it has been suggested that immunocytes could play a role in the vectorial capacity of insects leading to an increased interest towards primary immunocyte cultures. We analysed at molecular and cellular level the immune response of the leafhopper *Euscelidius variegatus* with the aim to provide an *in vitro* model for studying the insect-microbe interactions. We *in vitro* cultured and kept alive for more than 3 months *E. variegatus* immunocytes that showed a mitotic capacity as well as adhesion and phagocytic activities. *In situ* hybridization revealed that the defensin gene is actively transcribed in cultured immunocytes, while cecropins were not recorded in this species. These promising results obtained with *E. variegaus*, a leafhopper frequently used as a laboratory experimental model of insect vector of phytoplasmas, will help in developing *in vitro* tools for the study of the interactions between these pathogens and their vectors.

Key Words: innate immunity; circulating immunocytes; defensin, insect vectors; leafhopper

Introduction

Insects, as well as all multicellular organisms, are surrounded by many prokaryotic and eukaryotic microorganisms, playing different roles from beneficial to pathogenic. Contrarily to vertebrates, insects rely on innate immunity only to defend themselves against pathogens (Lavine and Strand, 2002). However, beside entomopathogens, many other microorganisms may inhabit the insects' body, such as mutualists, commensals and saprophytes (Douglas, 2014). These microorganisms, which are differentially related to insects, exhibit a range of interactions with their immune system. For instance, obligate intracellular endosymbionts, which are strictly coevolved with their hosts, undergo strong genome reduction due to selective pressure that leads to the loss of many genes encoding target molecules of insect immune receptors; therefore they are able to escape defence responses (Login and Heddi, 2013). On the other hand, many endosymbionts have been reported to induce an immune response (Nakabachi *et al.*, 2005; Anselme

et al., 2006; Ratzka *et al.*, 2011). Facultative symbionts as well have developed different strategies to escape or modulate insect mechanisms of immune response (Eleftherianos *et al.*, 2013), even often sharing with pathogens many molecular pathways recognized by the insects' immune receptors (Login and Heddi, 2013). Insect vectored plant pathogens, instead, were shown to either elicitate or suppress the immune responses (Eliautout *et al.*, 2016; Vyas *et al.*, 2015). In addition, insects regulate their immune response to control microbial infections by up- or down-regulating immune related genes in response to symbiotic microorganisms to maintain the microbiota balance (Wang *et al.*, 2009; Login *et al.*, 2011; Weiss and Aksoy, 2011; Eleftherianos *et al.*, 2013), avoiding detrimental immune induction which could result in disease-related dysbiosis (Buchon *et al.*, 2013).

Microbe-microbe interaction within the host's body may be mediated by the insect immune system as well and some microorganisms can promote or depress the growth of another ones by eliciting the host production of immune-active molecules (Douglas, 2014). This immune stimulation can be beneficial for the insect by contributing to the protection from pathogens and it may lead to the elimination of other harboured non-
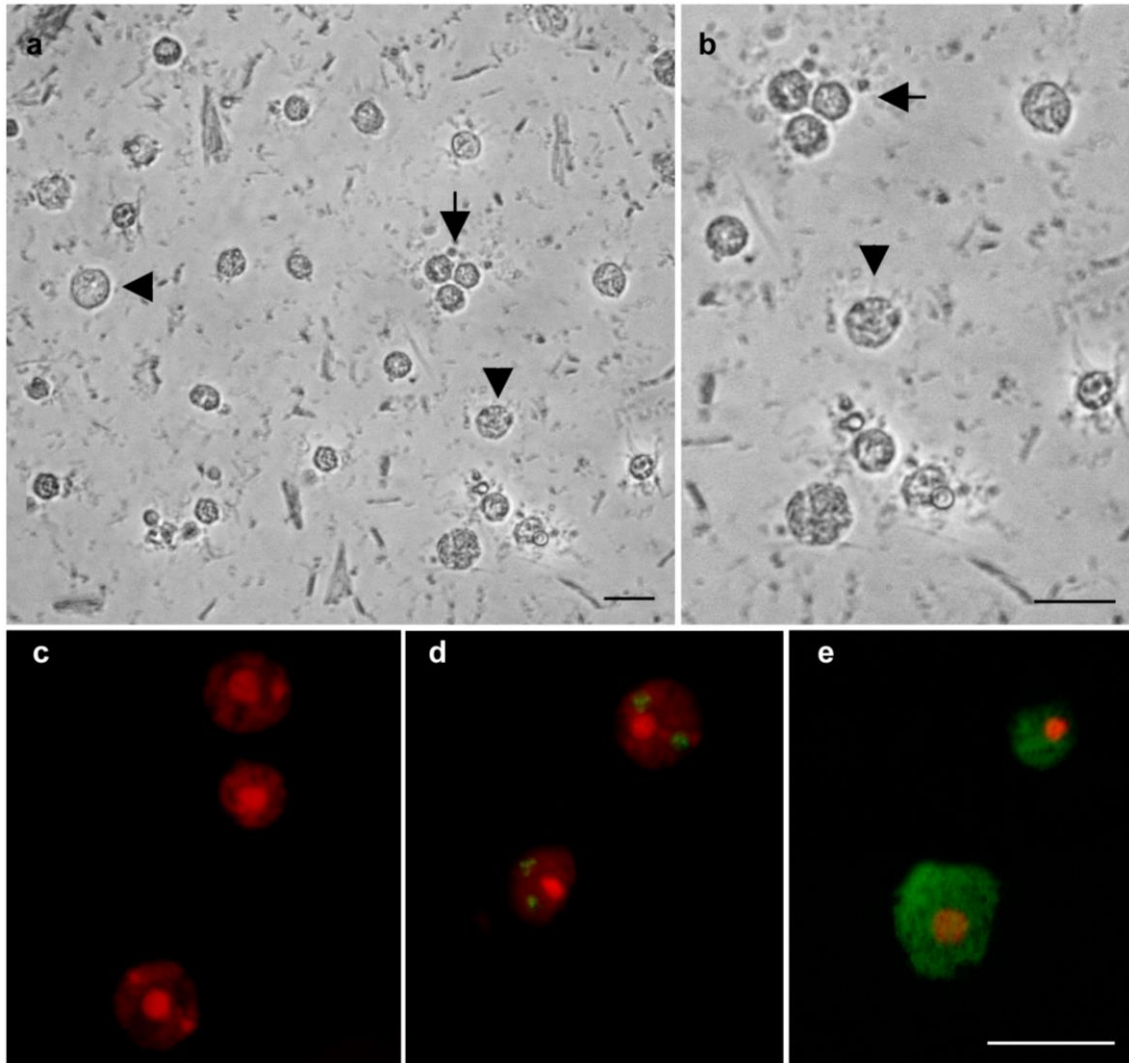
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Fig. 1 Observation of *E. variegatus* immunocytes at the inverted microscope with a 20X (a) and 40X (b) phase contrast objective showing small immunocytes resembling prohemocytes (indicated by arrows) and large cells with abundant cytoplasmic inclusions here referred as granulocytes (indicated by arrow heads). Both the immunocyte types resulted able to adhere to glass slides (c), phagocytize fluorescent microspheres (d) and express defensins (e). Cells in photographs c, d and e have been counterstained with propidium iodide. Bars = 10 μm.

symbiotic microorganisms, including vector-transmitted disease agents (Weiss and Aksoy, 2011). In these cases, the immune response induction may be favourable for the insects because, even though the role of human or plant pathogens on their insect hosts is poorly known, they may have detrimental effects for vectors' life span and fecundity (Hu *et al.*, 2008; Cassone *et al.*, 2014; Nachappa *et al.*, 2014; Alma *et al.*, 2015; Olson and Blair, 2015).

The insect innate immunity system is subdivided into humoral and cellular defense responses, with the production of antimicrobial peptides (AMPs) as a key process for both. AMPs are synthesized, predominantly, in fat body and released into hemolymph (Tsakas and Marmaras,

2010). Some AMPs, such as defensis and cecropins, are common and highly conserved in different insect orders, while some others are more sporadic. Insect defensins are active mainly against Gram-positive bacteria, even if some insect defensins are also active against the Gram-negative *Escherichia coli* and some fungi. On the contrary, cecropins have a broad spectrum of activity against both Gram-negative and Gram-positive bacteria, as well as fungi (Yi *et al.*, 2014).

New studies on immunocyte-mediated immunity highlighted the importance of cellular immune responses (Tsaka and Marmaras, 2010; Goto and Kurata, 2012). Different types of immunocytes (frequently referred as hemocytes) can be found circulating in the insect hemolymph (Marmas and

Lampropoulou, 2009). They are produced during embryogenesis and within juvenile stages, and react to attack by pathogenic agents with different responses, including phagocytosis, encapsulation, melanization and production of antimicrobial peptides, as well (Mandrioli *et al.*, 2003; Strand, 2008; Buchon *et al.*, 2014). Recently, immunocytes have been shown to interact with symbionts and play a role in the vectorial capacity of insects (Mandrioli, 2009; Mandrioli *et al.*, 2015a). As a consequence, the establishment of primary cultures of immunocytes has been widely increased in the last decades as a useful tool for the characterization of the immune response in different insects (Mandrioli *et al.*, 2015b). In particular, primary immunocyte cultures of insect vectors of plant pathogens has been proposed as new tools for studying the interactions between the pathogen and the host as well as the interplay between symbionts

and immunocytes, leading to a better comprehension of insect vector competence (Monti *et al.*, 2014).

Here we analyse, at molecular and cellular level, the immune response of the leafhopper *Euscelidius variegatus*, with aim to provide an *in vitro* model for studying insect immune response within the order Hemiptera, with special regard to insect-microbe interactions. *E. variegatus* can be considered an optimal leafhopper model for such studies because, besides harbouring facultative symbionts (Degnan *et al.*, 2011), it is a vector of plant pathogens in the 'Candidatus Phytoplasma' genus, namely Chrysanthemum Yellows Phytoplasma ('*Ca*. Phytoplasma asteris) and Flavescence dorée phytoplasma, and it was widely used as model species to study insect-phytopathogen interactions (Bressan *et al.*, 2005; Bosco *et al.*, 2007; Rashidi *et al.*, 2014).
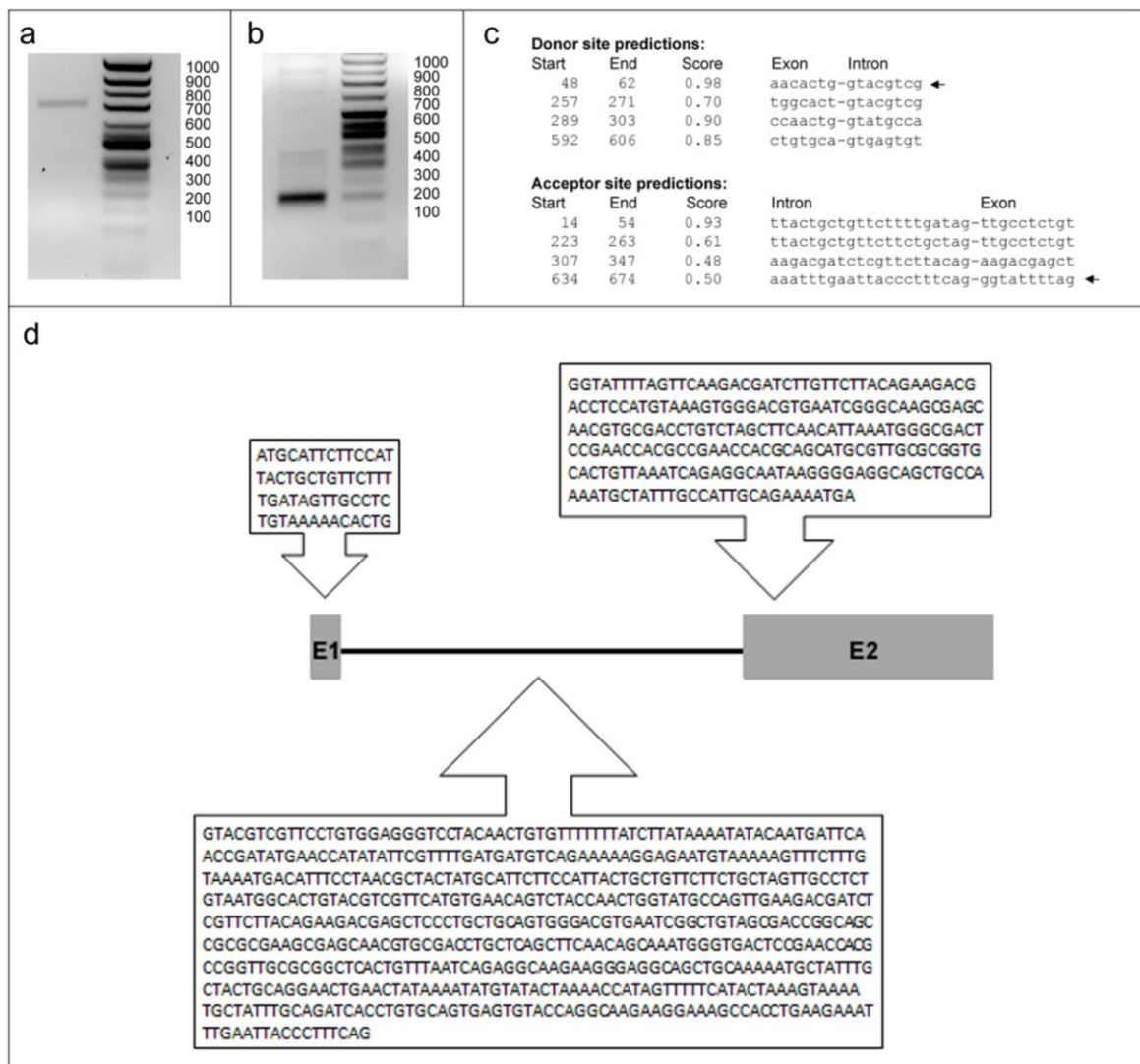
Fig. 2 Amplification of the *E. variegatus* gene from genomic DNA (a) and cDNA (b) samples evidenced a 54 bp long exon 1 and a 225bp long exon 2 separated by a 601 bp intron (d). The presence of the intron was confirmed by splice site prediction (c) (indicated by arrows).

Materials and Methods

E. variegatus *primary cell cultures*

Adults of *Euscelidius variegatus* were obtained from laboratory lines reared on oats (*Avena sativa* L.) in climatic chambers with 20- 25 °C and a photoperiod of 16:8 (L:D) hours (h), at the Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA). They were used to establish cell cultures, according to Monti *et al.* (2014). In particular, two female adults were washed in 0.115 % sodium hypochlorite, 75 % ethanol and MilliQ sterile water for 10, 30 and 20 seconds (sec), respectively. After drying on a filter paper for a couple of seconds, they were put in a single well of a sterile 24-well cell culture plate (Costar®, Corning, NY, USA) containing 1 ml of Hert-Hunter 70 (HH70) medium (Marutani-Hert *et al.*, 2009). The HH70 medium has been supplemented with 10 ml/L L-glutamine 200 mM solution (Invitrogen, CA, USA), gentamicin (at a final concentration of 50 µg/ml, Sigma-Aldrich, MO, USA) and penicillin/streptomycin (Sigma-Aldrich, MO, USA) at a final concentration of 50 U/ml at 50 µg/ml, respectively. The antimycotic agent nystatin (Sigma-Aldrich, MO, USA) was also added to each medium at a final concentration of 100 U/ml.

Plates were incubated at 24 – 26 °C and 0.2 ml of medium was added every 48 h if necessary, whereas the observation of cell cultures and the evaluation of the cell growth were carried out daily using an inverted Leica DMI3000 light microscope.

Functional assays by adhesion test and phagocytosis assay

An aliquot of 200 µl from each immunocyte cell culture was collected and placed on a glass slide in an aseptical Lab-Tek Chamber Slide system (Nunc, Naperville, IL, USA). Immunocytes were let settled for 30 minutes (min) in presence of HH70 medium. Thereafter, the slide was removed from the chamber slide system, stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence microscope. Photographs were taken using a CCD camera as previously reported. For each cell culture, a phagocytosis assay was performed. Briefly, a 200 µl aliquot was sampled and added to 100 µl of HH70 medium in a 0.2 ml tube previously covered and the material was then incubated with 0.1 µl of a fluorescent beads suspension for 30 min in soft oscillation, according to Manfredini *et al.* (2008). After incubation, cells were cytocentrifuged onto glass slides, stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence microscope.

Search for defensin and cecropin coding genes

Genomic DNA extraction was performed using the "SV Genomic DNA Purification System" (Promega, WI, USA) according to the manufacturer's instructions. PCR amplification of an internal portion of the defensin gene was carried out using the primers *EvDef* F (5'-ATGCATTCTTCCATTACTGCTG) and *EvDef* R (5'-CAGCTGCCTCC CTTCTTGC). Primers were selected by aligning the defensin coding sequences

of the hemipterans *Triatoma brasilensis* (ACH57151), *Nilaparvata lugens* (AGK40896) and *Rhodnius prolixus* (AAO74624) available in GenBank. The amplification mix contained 100 ng of genomic DNA, 1 mM of each primer, 200 mM dNTPs and 2U of DyNAZyme II DNA polymerase (Finnzymes Oy, Finland). Amplification was performed with a thermal cycler at an annealing temperature of 54 °C for 60 sec and extension at 72 °C for 60 sec. RNA extraction was performed with the "SV Total RNA Isolation System" (Promega), accordingly to the supplier's suggestions. In order to complete the defensin coding gene identification, a rapid amplification of cDNA ends (RACE) was performed following the method of Frohman (1990). The amplified fragments were cloned with the "pGEM T-easy cloning kit" following the Promega protocols. RACE has been performed on cDNA samples obtained by the total RNA, extracted using TRI-REAGENT TM (Sigma) following the method described by the supplier, and the successive reverse transcription using oligo-dT primers and the Access RT-PCR System (Promega), according to the supplier's protocols. PCR search for cecropin coding genes was carried using the primers *EvCec* F (5'- ATTGGACAATCGGAAGCTGG) and *EvCec* R (5'- CAGTTGCGGCGACATTNG), designed after the alignment of the cecropin coding genes of the insects *Drosophila melanogaster* (AAF57025), *Ceratitis capitata* (XP_004534334) and *Bombyx mori* (NP_001037392). The choice of using those insects in place of hemipteran species is due to the absence of cecropin genes in the currently studied hemipteran species. Sanger sequencing was performed at the BMR Genomics (Padua, Italy), whereas sequence analysis was carried out using the CLC Sequence Viewer Software (Madison, WI, USA) and using the *Splice Site Prediction* (freely available at the address: http://www.fruitfly.org/seq_tools/splice.html) (Reese *et al.*, 1997). The *E. variegatus* defensin gene sequence can be retrieved from GenBank under the accession number KX198127.

Semi-quantitative analysis by Reverse Transcription PCR (RT-PCR) of antimicrobial peptide (AMPs) expression in vitro

In order to study the induction of defensin after bacterial challenges, *E. variegatus* immunocytes were incubated with a 10^9 cells/ml *Staphylococcus aureus* (Gram positive), *Escherichia coli* (Gram negative) and *Asaia* sp. solutions for 0, 3, 6, 9, 12 and 24 h. After treatments (repeated in triplicates for each challenge), cells were centrifuged at 800*g* for 5 min at room temperature and the supernatant was discarded. RNA extraction was performed with the "SV Total RNA Isolation System" (Promega), accordingly to the supplier's suggestions. After extractions, RNA quality and concentration were assessed with a ND-1000 spectrophotometer (NanoDrop, DE, USA). RT-PCR has been performed with the Access RT-PCR System (Promega), according to the supplier's protocols. Cytoplasmic actin was amplified (primers actF 5'-AGCAGGAGATGGCCACC-3' and actR 5'-TCCACATCTGCTGGAAGG-3') as a loading control, according to Capone *et al.* (2013). For the

cytoplasmic actin, PCR reactions (20 cycles), the following parameters were used: annealing temperature 58°C; annealing time 40 sec, elongation time 45 sec. RT-PCR amplification was evaluated by electrophoresis in 1.2 % agarose gel (run 100V for 45 min). Gel documentation was collected using a "Gel Doc XR", digitally evaluated with "Quantity One" (Bio-Rad Lab, Milano, Italy) and normalized to the correspondent signals for cytoplasmic actin. Three replicates were carried out for each induction.

In situ *hybridization*

The presence of defensin mRNA in the *E. variegatus* immunocytes was studied using a defensin probe labelled with digoxigenin (DIG) by end-labelling procedure (Roche, Switzerland). The *in situ* hybridization assay was performed using a non-radioactive procedure. Briefly, immunocytes were cytocentrifuged at 800 rpm for 3 min. Split cells were then fixed in PBS buffer containing 4 % paraformaldeyde and then permeabilized with PBS buffer containing 0.3 % Triton X-100. Cells were incubated with labelled probes for 16 hours at 42°C and subsequently washed at 42 °C in SSC 2X and 1X, respectively. After 30 min incubation with normal serum, cells were incubated with a fluorescein-conjugated anti-DIG antibody for 2 h in the dark. Nuclei were counterstained using a 100 ng/ml propidium iodide solution for 5 min at RT.
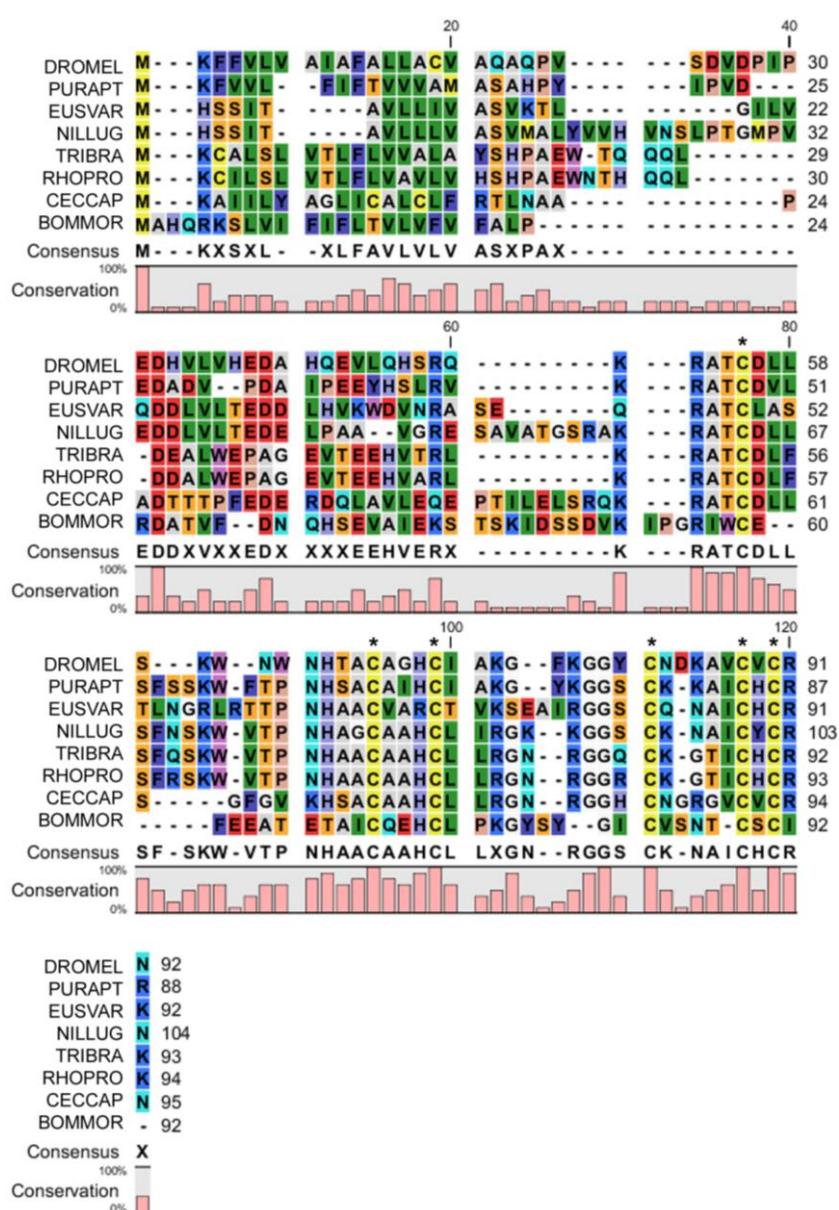
Fig. 3 Alignment of the defensin aminoacidic sequences from *Drosophila melanogaster* (DROMEL), *Pyrrhocoris apterus* (PURAPT), *Triatoma brasilensis* (TRIBRA), *Nilaparvata lugens* (NILLUG), *Rhodnius prolixus* (RHOPRO), *Ceratitis capitata* (CERCAP) and *Bombyx mori* (BOMMOR) revealed that *E. variegatus* (EUSVAR) defensin is well conserved, including the presence of six highly conserved cysteine residues (indicated by asterisks).

Results

On the basis of previous successful results with hemipteran immunocytes (Monti *et al.*, 2014), we isolated and *in vitro* cultured *E. variegatus* immunocytes in the HH70 medium with positive results and we kept cells alive for more than three months (Fig. 1). Cell counts showed a slightly declining cell number in the first 15 days with mitoses observed in plates starting after 12 days of *in vitro* maintenance.

Most of the observed immunocytes were small in size with the nucleus occupying the central part of the cellular body and they resembled typical insect prohemocytes (Figs 1a-b). A second type observed consisted of cells larger than the previous with abundant cytoplasm containing cytoplasmic inclusions varying in shape from round to irregular or elongated that have been generally referred as granular cells (Figs 1a-b).

Adhesion tests showed that *E. variegatus* immunocytes were able to adhere to a glass slide after 30 min incubation (Fig. 1c). Moreover, 72 % of cells were able to phagocytize fluorescent microspheres assessing that they are functional despite their *in vitro* maintenance (Fig. 1d).

PCR amplification with the defensin primers evidenced a product of about 800 bp using genomic DNA as a template (Fig. 2a) and an approximately 200 bp amplicon using cDNA samples (Fig. 2b); these results, as a whole, clearly suggest the presence of an intron. RACE PCR allowed the amplification of both the defensin cDNA termini, allowing the identification of the complete defensin coding sequence in *E. variegatus*.

Sequence analysis revealed that the defensin gene consists of a 54 bp long exon 1 and a 225 bp long exon 2 separated by a 601 bp intron (Fig. 2d). The presence of the intron was also confirmed by bioinformatic analyses performed using the *Splice Site Prediction* (Fig. 2c).

The *E. variegatus* defensin consists of 92 aminoacidic residues exhibiting a 36 % to 60 % similarity with other insects, with an increase to 50 - 60 % if the comparison is limited to hemipteran defensins (Fig. 3). Sequence alignment also showed a conserved localization of 6 cysteine residues (generally referred as cysteine 3 - 8 in the defensin peptide) suggesting their involvement in the formation of three disulfide bridges in the *E. variegatus* defensin (Fig. 3).

In situ hybridization revealed the defensin gene was transcribed in the *in vitro* cultured immunocytes evidencing a bright FITC fluorescence in the cytoplasm (Fig. 1e).

The identification of the defensin gene allowed us to study its induction after bacterial challenges on *in vitro* cultured *E. variegatus* immunocytes (Fig. 4). In particular, RT-PCR experiments clearly showed that *S. aureus* only induced the defensin gene expression after 6 h, whereas no induction has been observed after *E. coli* and *Asaia* challenge (Fig. 4).

PCR amplification with the cecropin primers evidenced a 160 bp product using cDNA samples obtained from the dipterans *D. melanogaster* and
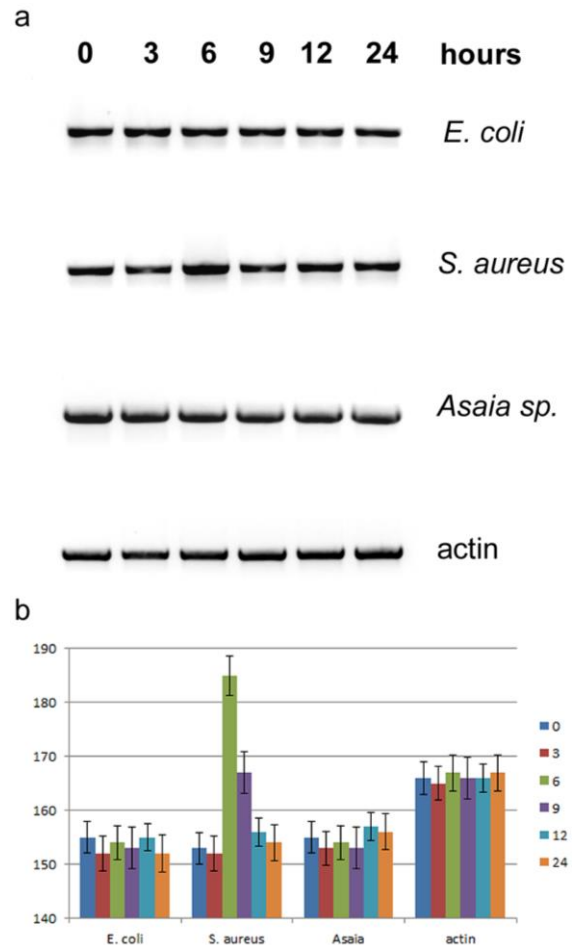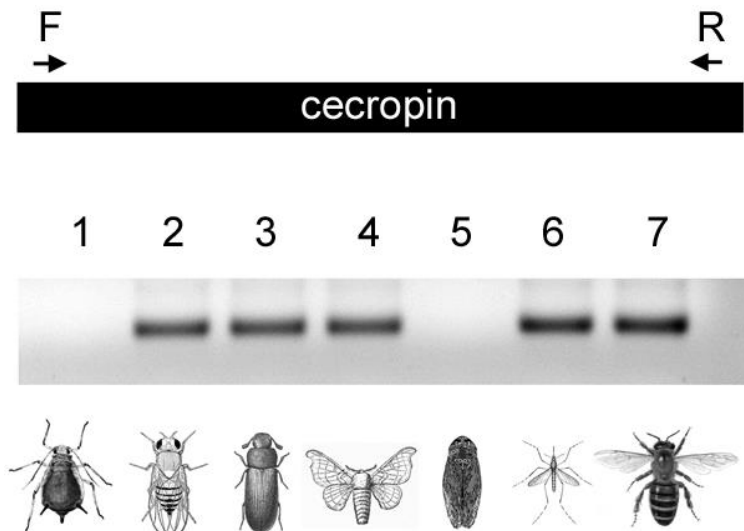

Fig. 4 a) RT-PCR amplification of the *E. variegatus* defensin from immunocyte samples challenged with *E. coli*, *S. aureus* and *Asaia* for times ranging from 0 to 24 h evidenced that defensin is constitutively expressed in *E. variegatus*, but its expression increased after a 6 h long exposure to the Gram-positive *S. aureus*. Cytoplasmic actin has been amplified for each sample as loading control. b) Graphical representation of the results obtained from experiments clearly evidences the induction of the defensin expression after exposure to the Gram-positive *S. aureus*.

Anopheles stephensi, the coleopteran *Tribolium castaneum*, the lepidopteran *B. mori*, and the hymenopteran *Apis mellifera* as a templates, whereas no amplification has been obtained in the hemipterans *Acyrthosiphon pisum* and *E. variegatus* (Fig. 5).

Discussion

Immunocytes play multiple functions in insects, including defence mechanism like nodule formation, phagocytosis, encapsulation and synthesis of antimicrobial peptides and other molecules (Pandey

Fig. 5 RT-PCR amplification of cecropin cDNA in *Acyrthosiphon pisum* (1), *D. melanogaster* (2), *Tribolium castaneum* (3), *B. mori* (4), *E. variegatus* (5), *Anopheles stephensi* (6) and *Apis mellifera* (7) assessed the absence of cecropin hortologues in both the analysed Hemiptera.

and Tiwari, 2012). Interestingly, in the last years a role of immunocytes in the interaction with symbionts and in the vectorial capacity of insects has been suggested making their study a big challenge for the scientific community (Mandrioli, 2009; Mandrioli *et al.*, 2015a). Indeed, the immunocyte study is not simply related to immunity, but also to future applications in applied fields related for instance to the biocontrol of insects involved in the spread of plant diseases.

Up to date, immunocytes have been studied in several insect species and in particular in Lepidoptera, where from 2 to 7 different cell types have been accurately described using morphological, histochemical and functional characteristics (Chauvin, 1968; Brehélin *et al.*, 1978; Brehélin and Zachary, 1983, 1986; Ahmad, 1992; Butt and Shields, 1996; Hernandez *et al.*, 1999; Manfredini *et al.*, 2008).

In view of the large number of diverse immunocyte types (including their different ultrastructures, size and distribution in the insect body), it has been suggested the "multiple-cell theory" about hematopoiesis and hemocyte differentiation, which suggests the existence of separate immutable cell lines, each differentiating from a single germinal stem, that give rise to the different hemocyte types (Akai and Sato, 1973; Gupta, 1985).

In our current analysis, we recognized in *E. variegatus* two types of circulating immunocytes due to their substantial uniformity in the cellular morphology, in agreement with previous observation in Hemiptera (Monti *et al.*, 2014). In particular, it seems that in *E. variegatus* the unique feature that marks distinctively a precise hemocyte variety is the presence of cytoplasmic granules, which are a prerogative of granular immunocytes. The other cells (even if they may be slightly different in size)

are generally agranular and with a large nucleus. In the case of *E. variegatus* no other feature seems to justify the identification of further immunocyte types. As a whole, in view of the small size, the thin cytoplasm, which is a proof for a cell that is beginning its growth, and the absence of phagolysosomes or granules, we considered the small, agranular cells as prohemocytes, whereas the second cell type observed in *E. variegatus* can be referred as granular cells or granulocytes.

Literature data on Hemiptera immunocytes are not abundant, but a previous study identified by phase-contrast microscopy seven morphological hemocyte types (prohemocytes, plasmatocytes, granular cells, cytocytes, oenocytoids, adipohemocytes and giant cells) in the species *Rhodnius prolixus*, *Rhodnius neglectus*, *Triatoma infestans*, *Panstrongylus megistus* and *Dipetalogaster maximus* (Azambuja *et al.*, 1991). Actually, not all of them are present in the different studied species: for example, adipohemocytes and oenocytoids were not observed in *P. megistus* and *P. infestans*, while giant cells were rarely found in any of the species studied.

Our proposal to refer to two type only is not unusual in literature, since also in honeybees and wasps few immunocyte types have been described (Chauvin, 1968; Manfredini *et al.*, 2008) supporting a more unitarian interpretation of the cellular elements of hemolymph. This approach supposes that the various hemocyte types are merely stages, with separate functions, of a single cell line derived from a unique germinal stem *i.e.,* the prohemocyte (Ottaviani, 2005; Manfredini *et al.*, 2008). The "single-cell theory" relies therefore on the presence of transitional stages of some immunocyte types and on the existence of only prohemocytes and granular cells in tissue cultures and hematopoietic organs (Lavine and Strand, 2002). This is in line

with the great functional versatility of these complex and highly specialized cells, which is required in order to achieve a wide physiological flexibility that is necessary to undergo ready transformation in response to environmental stimuli and/or bacterial challenges (Ottaviani, 2005; Manfredini *et al.*, 2008).

According to literature data, immunocytes can grow and multiply also *in vitro* for an indefinite period, without the involvement of hematopoietic organs (Gupta, 1985). Even if with a low mitotic index, *E. variegatus* circulating immunocytes showed a mitotic capacity, suggesting that these prohemocytes are able of dividing not only as stem cells, as suggested in other insects (Brehélin and Zachary, 1986; Franchini *et al.*, 1996), but also when already circulating in the hemolymph. This aspect could be very important taking into account that immunocytes could interact with different symbionts and/or plant pathogens that can move within the insect body, where the number of immunocytes could be relevant for a proper immune response.

Molecular analyses and FISH clearly showed that *E. variegatus* immunocytes are able to synthesize antimicrobial peptides belonging to the defensin family that are active against Gram-positive bacteria. This result was not unexpected since defensins form a family of antibacterial peptides that is widely distributed in insects (Bulet *et al.*, 1999; Lamberty *et al.*, 1999), including the presence of defensins expressed in insect cell lines (Fallon and Sun, 2000).

As a whole, our results suggest the presence in *E. variegatus* of a gene coding for defensins, but the absence of genes coding for cecropins, in accordance to data obtained from the genome and transcriptome analyses of the brown planthopper *N. lugens* (Bao *et al.*, 2013). The absence of genes encoding antimicrobial peptides that are common in other insects, including defensins and cecropins, is not unusual in Hemiptera since cecropins and defensins are also missing in aphids, as reported in the pea aphid *A. pisum* (The International Aphid Genomics Consortium, 2010).

These results are very useful in order to verify the chance of developing *in vitro* tools for the study of the interaction between phytoplasmas and the host insect vectors using *E. variegatus* as an experimental model. In order to plan *in vitro* analyses, it is essential to know the presence/absence of the most common molecules involved in the immune response, such as defensins and cecropins to verify if phytoplasmas may induce or modulate the insect immune response. Indeed, as revised by Bosco and D'Amelio (2010), once in the hemocoel of the insect vector, phytoplasmas may circulate and multiply in the body cavity, and pass through the salivary glands before being excreted together with hemipteran saliva during successive feeding events. The innate immune system of insect vectors could therefore play a major role in enabling phytoplasma multiplication in and colonization of the insect body. This would be useful for clarifying the detailed physiological and immunological mechanisms in *E. variegatus* and could provide potential targets for the management of leafhopper phytoplasma vectors in the future.

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