Journal of Insect Physiology 59 (2013) 1095–1103

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Identification and characterization of *defensin* genes from the endoparasitoid wasp *Cotesia vestalis* (Hymenoptera: Braconidae)

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ARTICLE INFO

Article history:
Received 4 June 2013
Received in revised form 24 August 2013
Accepted 26 August 2013
Available online 5 September 2013

Keywords:
Defensin
Endoparasitoid wasp
Developmental stage
Innate immunity
Antimicrobial peptide
Antimicrobial activity

ABSTRACT

Defensins are members of a large and diverse family of antimicrobial peptides (AMPs) containing three or four intramolecular disulfide bonds. They are widely distributed from vertebrates to invertebrates, and serve as critical defense molecules protecting the host from the invasion of pathogens or protozoan parasites. *Cotesia vestalis* is a small endoparasitoid wasp that lays eggs in larvae of *Plutella xylostella*, a cosmopolitan pest of cruciferous crops. We identified and characterized three full-length cDNAs encoding putative defensin-like peptides from *C. vestalis*, named *CvDef1*, *CvDef2* and *CvDef3*. Phylogenetic analyses of these sequences showed that they are present in two clades, CITDs and PITDs, indicating a diversity of defensins in *C. vestalis*. We analyzed their expression patterns in larvae, pupae and adults by semi-quantitative RT-PCR. The results showed that *CvDef1* mRNA was expressed from the end stage of the second instar larva, *CvDef3* mRNA from the early stage of the second instar larva, and *CvDef2* mRNA was expressed in all developmental stages of *C. vestalis*. Furthermore, CvDef1 showed antimicrobial activity against gram-positive and gram-negative bacteria. Growth kinetics of *Staphylococcus aureus* indicated that CvDef1 had much better antimicrobial ability than ampicillin, making it a potential candidate for practical use. Transmission electron microscopic (TEM) examination of CvDef1-treated *S. aureus* cells showed extensive damage to the cell membranes. Our results revealed the basic properties of three defensins in *C. vestalis* for the first time, which may pave the way for further study of the functions of defensins in parasitism and innate immunity of *C. vestalis*.

1. Introduction

Antimicrobial peptides (AMPs) are members of a large family of peptides that possess antibacterial and antifungal activities (Auvynet and Rosenstein, 2009). AMPs have redefined the way we think about immune defense since the first cecropins were isolated and characterized from hemolymph of the moth *Hyalophora cecropia* (Hultmark et al., 1980). To date, more than 1200 AMPs have been identified or predicted from various organisms. AMPs are small (12–50 amino acids) peptides that are positively charged and possess relatively hydrophobic and amphipathic structures that are thought to interact with microbial membranes (Wimley, 2010). AMPs are now known to exist in all kingdoms and are considered a key element of innate immunity due to their pleiotropic functions to not only kill microbes but also to control host physiological functions such as inflammation, wound repair and immunomodulation (Auvynet and Rosenstein, 2009; Hancock et al., 2006; Nakatsuji and Gallo, 2012).

Defensins are diverse in their primary sequences, but possess a characteristic cysteine-rich region structured with three or four intramolecular disulfide bonds. On the basis of the positions of the six conserved Cys residues and the amino acid sequence, members of this family of peptides have been classified into α-Defensins (present before placental and marsupial divergence), β-Defensins (considered the ancestral gene type) and θ-Defensins (Ganz, 2003). β-Defensins and θ-Defensins are only specifically found in vertebrate, while the α-Defensins are widely distributed in both vertebrates and invertebrates. However, although vertebrate and invertebrate α-Defensins have similar antibacterial modes of action, they are considered to be phylogenetically unrelated due to differences in structure and sequence (Froy and Gurevitz, 2003). Insect defensins were first discovered in the flesh flies *Sarcophaga peregrina* and *Phormia terranovae* (Lambert et al., 1989; Matsuyama and Natori, 1988). Since then, more than 70 defensins have been identified from arthropods belonging to diverse taxa such as insects, ticks, spiders, and scorpions (Bulet et al., 2004). Previous studies have shown that defensins possess broad-spectrum antimicrobial activity against bacteria, fungi, and enveloped viruses, properties that make defensins excellent candidates for solving

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http://dx.doi.org/10.1016/j.jinsphys.2013.08.011

the increasing problem of microbial drug resistance (Pasupuleti et al., 2012).

Parasitoid wasps are insects whose larvae parasitize various life stages of other arthropods (for example, insects and spiders). Genome sequencing of three *Nasonia* (Pteromalidae) species, all ectoparasitic hymenoptera, provide a new resource to study the molecular mechanism of innate immunity in parasitoid wasps (Werren et al., 2010). Since then, a number of defensins have been identified from *Nasonia* using a combined computational and experimental strategy (Gao and Zhu, 2010; Tian et al., 2010a; Ye et al., 2010). Two different subtypes of defensins (defensin1 and defensin2) were described in the *Nasonia* lineage, and only defensins from subtype 1 were constitutively expressed in the *Nasonia vitripennis* adult stage and were up-regulated after bacterial infection (Gao and Zhu, 2010). A defensin-like peptide, Defensin-NV, was purified and characterized from venom of *N. vitripennis* which exerted strong antimicrobial activity against tested microorganisms including Gram-positive bacteria, Gram-negative bacteria and fungi (Ye et al., 2010). However, the distribution of defensins in endoparasitoid wasps is not known. *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae) is a small endoparasitoid wasp which lays eggs in larvae of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), a cosmopolitan pest of cruciferous crops. It employs multiple factors such as venom, polydnaviruses (PDVs) and teratocytes in active and passive immune-suppression to overcome the defenses of its host (Beckage and Gelman, 2004). We hypothesize that defensins play a pivotal role in innate immunity of this insect as they do in other animals. Here we characterized three defensin-encoding genes from the endoparasitoid wasp *C. vestalis* and evaluated the antimicrobial activities of their gene products.

2. Materials and methods

2.1. Insect rearing and parasitization

C. vestalis and its host *P. xylostella* were reared as previously described (Chen et al., 2011). Both were maintained at 25 ± 1 °C, 65% relative humidity under a 14:10 h (L:D) photoperiod. Adults were fed 20% honey/water (V/V). Late second instar *P. xylostella* larvae were individually exposed to a single *C. vestalis* female within a 10 mm × 80 mm test tube to ensure 100% parasitization. Each *P. xylostella* larva was then removed from the test tube and reared on cabbage. Adults of *C. vestalis* that emerged from the cocoons were collected, allowed to mate for 24 h, and then used for parasitization.

2.2. RNA isolation and rapid amplification of cDNA ends (RACE)

Total RNA was extracted from *C. vestalis* with TRIzol reagent (Invitrogen) and RNA yield was determined by NanoDrop 2000 (Thermo, USA). 3′ and 5′ RACE were performed with the SMART RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's instructions. Primer sequences used in this research are shown in Table 1. All PCR-amplified DNA fragments were cloned into pMD-19 vectors (Takara, Japan) and then sequenced.

2.3. Sequence and phylogenetic analysis

Database searches were performed with Blastx (http//www.ncbi.nlm.nih.gov/). Translation of the cDNA and prediction of deduced peptides were performed with ExPASy Translate Tool (http://web.expasy.org/translate/). Signal sequences were predicted by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and structural prediction was undertaken with SWISS–MODEL (http://swissmodel.expasy.org/). The translated amino acid sequences of defensins from insects were aligned using the CLUSTAL X program. The phylogenetic analysis was carried out running MrBayes Section 3.1.2 (Ronquist and Huelsenbeck, 2003). Alignment gaps and missing data were eliminated in pairwise sequence comparisons. The Markov chain Monte Carlo searches were run with four simultaneous chains for 5,000,000 generations, with trees being sampled every 5000 generations. The first 250 trees were discarded as "burnin", keeping only trees generated well after those parameters stabilized.

Table 1
Primer sequences used in this paper.

Primer name	Primer sequences (5′–3′)	Use
CvDef1-for 1	TATTATTTTAAAAGTTTTACGATGGCC	3′ RACE
CvDef1-for 2	TTCGTTTGCCACGGGCTACCTG	3′ RACE
CvDef1-rev 1	GATATATAAATTATCCATTAAATGC	5′ RACE
CvDef1-rev 2	AGCCCGTGGCAAACGAAAATG	5′ RACE
CvDef2-for 1	TTCTCCAAAAAATGAAGTCCAAGC	3′ RACE
CvDef2-for 1	AAAGGAGGACACTGTGGAAG	3′ RACE
CvDef2-rev 1	TTTATGATTGGTCGCTCAGAAG	5′ RACE
CvDef2-rev 2	TCAATCAGGATGGTGTTTCA	5′ RACE
CvDef3-for 1	ATTTTGTCAGTGATTGAGAAGA	3′ RACE
CvDef3-for 1	ACATTCAAAGCACTTCCAC	3′ RACE
CvDef3-rev 1	TTCCGCAGTGTCCGCCTTTG	5′ RACE
CvDef3-rev 1	GCAGTTTGAAGTGTAGTTGGTGGC	5′ RACE
qCvDef1-for	TATTATTTTAAAAGTTTTACGATGGCC	RT-PCR
qCvDef1-rev	ATCCATTAAATGCTATTCGCCA	RT-PCR
qCvDef2-for	GAGTACATGGGGAGTTAACTTC	RT-PCR
qCvDef2-rev	CAGCACACGTGTCAATTG	RT-PCR
qCvDef3-for	ATTTTGTCAGTGATTGAGAAGA	RT-PCR
qCvDef3-rev	AACAAAATTCGAATGCCTC	RT-PCR
18sRNA-for	ATGTCTGCCTTATCAACTGTCG	RT-PCR
18sRNA-rev	TCCTTGGATGTGGTAGCCG	RT-PCR

2.4. Genomic organization

Genomic DNA was extracted from a single adult using a DNeasy tissue kit (Qiagen, Hilden, Germany) following manufacturer's protocols. Standard PCR was carried out using LA Taq polymerase (Takara, Japan). The full-length *defensin* gene was amplified from the *C. vestalis* DNA with specific primers. The amplification program consisted of an initial denaturing step (95 °C for 5 min), followed by 30 cycles of denaturing (94 °C for 30 s), annealing (56 °C for 25 s), and extension (72 °C for 1 min), and finally elongated at 72 °C for 10 min. The products were then cloned and sequenced.

2.5. Detection of developmental stage-specific expression of defensins

Specific primers based on the three defensin sequences were designed to distinguish and amplify their corresponding cDNAs. To prepare total RNAs of *C. vestalis*, the pupae, adults (female and male) and larvae of different instars dissected from parasitized *P. xylostella* were collected separately and subjected to an SV Total RNA Isolation System (Promaga, China). cDNAs were synthesized using the ReverTra Ace qPCR RT kit (TOYOBO, Japan) after RNA samples were denatured at 65 °C. For semi-quantitative RT-PCR (RT-PCR), *C. vestalis* 18sRNA was used as an internal control. The volume of each cDNA pool was adjusted to give the same amount of the 18sRNA product in the exponential phase. The amplification program was the same as those described above. The PCR products were taken for comparison of the amounts of these products by electrophoresis on 2% agarose gels.

2.6. Peptide synthesis

Three peptides derived from the three cDNA sequences were chemically synthesized by the Sangon Biotech (Shanghai, China), and their purity was confirmed by HPLC and MALDI–TOF (Table 2).