Gene Expression and Phylogenetic Relationship of Defensin Genes from Egyptian Sources for Biocontrol

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ABSTRACT

Defensins are subgroup of antimicrobial peptides that play an indespensible role in innate immunity in almost all eukaryotic animal and plant cells ranging from insects to mammals. They represent a wide range of structure and mode of action depending on their source. The main objective of this study is to isolate the defensin gene from various Egyptian sources to study the phylogenetic relationship among these organisms. Defensin genes were isolated from various Egyptian sources including *Sitophilus oryzae* (rice weevil), *Callosobruchus chinensis*, *Bos Taurus* (Egyptian and Friesian), *Homo sapiens* (human blood cells). Using PCR technology, various amplicons were amplified from the different organisms. An amplicon of 150 bp was amplified and isolated from *Sitophilus oryzae*, *Callosobruchus chinensis*. Amplicon of 429 bp was amplified from Egyptiam cows and three amplicons of 1650 bp, 1300 bp, and 429 bp were amplified from Friesian cows. Three amplicons of 800 bp, 215 bp, and 179 bp were amplified using primer hBD-1, two amplicons of 1000 and 179 bp were amplified using primer hBD-2. Sequences obtained from the amplified amplicons were used to construct a phylogenetic tree among the used organisms. The results confirm that defensins from Egyptian sources are highly diversed within the same species as well as among different species.

Key words: Defensins; PCR, DNA.

INTRODUCTION

Defensins are subgroup of antimicrobial peptides widely present in eukaryotic cells as part of innate immune system. Defensins are small cysteine-rich cationic proteins which are active against bacteria, fungi (Yamaguchi and Ouchi, 2012). They consist of 12-50 amino acids including six to 8 conserved cysteine residues. Cells of the immune system contain these peptides to assist in killing phagocytized bacteria. Most defensins function by binding to microbial cell membrane forming pore-like membrane defects that allow efflux of essential ions and nutrients (Alberts et al 2002; Lehrer and Ganz, 2002). More than 80 different defensins have been characterized and three subfamilies have been identified in mammals: α- defensins, β- defensins, and θ- defensins. Study of the three dimensional structure of a number of plant defensins have shown that the structure comprises a triple–stranded β–sheet with an α-helix in parallel (Bruix et al. 1993, 1995; Bloch et al. 1998; Fant et al. 1998; Almeida et al. 2002). At the beginning of the 1990s, the first plant defensins were isolated from wheat and barley grains (Colilla et al. 1990; Mendez et al. 1990). At that time these proteins were called γ thionins since their size and cysteine content were found to be similar to the previously called thionins (Carrascol et al. 1981). Plant defensins are small (45-54 amino acids) highly basic cysteine- rich peptides that are apparently ubiquitous throughout the plant kingdom. All plant defensins identified so far have eight cysteines that form four structure stabilizing disulfide bridges.

Plant defensin is small highly stable cysteine-rich peptides. They represent major part of the innate immune system against bacterial and fungal pathogens (Stotz et al., 2009). They do not interact directly with plasma membrane like mammalian and insect defensin (Stotz et al., 2009a; Wang et al., 2005; Caravalho et al., 2009). They play indespensible roles in protection of seeds and other plant organs, antimicrobial activity, regulation of plant growth and development (Stotz et al., 2009a; Stotz et al., 2009b). Moreover, plant defensins have a characteristic three-dimensional folding pattern that is stabilized by eight disulfide linked cysteines. In insect defensin, an α- helix is combined with a double– stranded β- sheet, stabilized by three disulfide bridges between six cysteine residues (Bonmatin et al. 1992; Cornet et al. 1995). Based on their secondary structure in aqueous solution or by sequence homology, they can classified to three main categories: (i) peptides belonging to the family of defensins with a α- helix/ β-sheet (αββ, βαββ) mixed structure, (ii) peptides with a triple stranded antiparallel β- sheets and (iii) peptides forming a hair pin like β sheet structure. The two pathogen-inducible peptides drosomycin and heliomicin isolated from the fruit fly *Drosophila meloanogaster* and from the lepidopteran *Heliothis virescens* (Landon et al. 1997; Lamberty et al. 1999).

Heliomicin, like insect defensins, while the structure of drosomycin, like plant defensins (Landon et al. 1997,; Lamberty et al. 1999).

Three types of defensins have been identified in mammals. A part from the 18 amino acids cyclic peptide only discovered in macaques and termed θ – defensin (Tang et al. 1999; Trabi et al. 2001). There are two forms of human defensins, α and β, are recognized, depending on the location and connectivity of the cysteines. α –Defensins have been localized to neutrophils and paneth cells of the intestine, whereas β- defensins are expressed by many epithelia (Ganz and Lehrer, 1995). Six human β defensins (hBD-1 through-6), have been identified. In addition to their broad spectrum antimicrobial properties, there is evidence that the β defensins act as chemokines for immature dendritic cells and memory T cells, and thus may serve as an important bridge between the innate and adaptive immune systems (Yang et al. 1999). In the human genome all known defensin genes cluster to a <1Mb region of chromosome 8p22-p23 (Harder et al., 1997; Liu et al., 1997; Linzmerier et al., 1999). The cDNAs and genomic sequences for HBD1 and HBD2 were identified after the peptides were first isolated from plasma or tissue sources using biochemical techniques (Bensch et al., 1995; Harder et al., 1997).

Defensins have a broad spectrum of antimicrobial activity, being effective against many Gram-positive and negative bacteria, some fungi and enveloped viruses. The antimicrobial activity of defensins has been attributed to permeabilization of microbial membranes and subsequent release of cellular contents. Two models have been suggested to achieve this. On one hand, defensin monomers assemble to form pores within the microbial membrane and, on the other hand, defensins disrupt the membrane by electrostatic interactions with the polar head groups of the bilayer. In addition to their antimicrobial effects, defensins have been shown to modulate a variety of cellular activities including chemotaxis of T cells, dendtritic cells, and monocytes, stimulation of epithelial cells and fibroblast proliferation, stimulation of cytokine production, and stimulation of histamine release from mast cells. These effects which typically occur at defensins concentrations much lower than those required for antimicrobial activity, suggest that defensins not only participate in the innate immune response system by virtue of their ability to kill microbes, but also that they act as a regulatory factors. The underlying genes responsible for defensin production are highly polymorphic. Some aspects are conserved, however; the the common features of a β-defensin are its small size, high density of cationic charge and six-cysteine-residue motif. Generally they are encoded by two-exon genes, where the first exon encodes for a hydrophobic leader sequence and the second for a peptide containing the cysteine motif. Five families of defensins have been reported in eukaryotes ranging from plants, insects, and mammals.

In this study, various organisms from Egyptian sources were employed to study the phylogenetic relationship among them and to search for antimicrobial peptides for biocontrol purpose.

MATERIALS AND METHODS

Materials

Insects: *Sitophilus oryzae, Callosobruchus chinensis*

These two insect species were obtained from Department of Entomology, Faculty of Agriculture, Alexandria University.

Bovine: *Bos taurus*

The animals were randomly chosen from the Faculty of Agriculture farm, Abeas, Alexandria. Blood samples for DNA analysis were collected from jugular vein by an authorized veterinarian to the tubes containing K_3 EDTA and stored at -80 ^{0}C.

Human: Human blood samples were collected from volunteers from medicine research institute, Alexandria University.

Primers: All the primers used in this study were synthesized by Metabion international AG Company, Deutschland. The nucleotide sequence of primers used in this study is shown in table (1).

Methods

DNA extraction from blood samples

Fifty µl of blood were added in 2 ml tube containing 700 µl from lysis buffer (10 mM Ttris–HCl, 100 mM NaCl, 1 ml EDTA pH8.0, 0.5% SDS) and 60 µg proteinase K was added and mixed well. After incubation at 37 ºC overnight or 65 ºC for 2 hours, equal volume of phenol: chloroform: isoamyl alcohol 25:24:1 was added mixed by inverting many times, and centrifuged at 12, 000 rpm for 10 minutes. The aqueous phase was transferred to a new tube. This step was repeated. Equal volume from chloroform: isoamyl alcohol was added and tubes were inverted many times, centrifuged at 12,000 rpm for 10 minutes. Two volumes of ethanol were added to the upper layer then samples were incubated in -80ºC for 30 minutes. Samples were centrifiuged; DNA pellet was washed with 70% ethanol, dried, and dissolved in 50 µl TE buffer.

Isolation of total RNA from insect

Ultra clean RNA isolation kit (MOBIO, USA) was used. 100 mg of tissues was homogenized in1ml of PMR1 buffer. The homogenate was transferred to a collection tube and the the samples were centrifuged at 1,000 g for 3 minutes. The homogenate was transferred to a new collection tubes and 500 µl of PMR2 buffer was added followed by 250 µl of PMR3.

Table 1: Nucleotide sequence of the primers used in this study.

N	Name of primer	Sequence 3' ⟶ 5'
1	BBD1S- F	'5 GCCAGCATGAGGCTCCAT 3'
2	BBD2A-R	'5 AACAGGTGCCAATCTGT 3'
3	IDG- F	'5 CCAAATGCCTCGTCATCT 3'
4	IDG- R	'5 ATTAGAGTCAAGCTCAAAAGGG-3'
5	HBD F	'5 ATGAGGATCCATTATCTTCT 3'
6	HBD3 R	'5 TTATTTCTTTCTTCGGCAGC 3'
7	HBD3'F	'5-TGTTTGCTTTGCTCTTCCTG-3'
8	HBD3'R	'5-CTTTCTTCGGCAGCATTTTC-3'
9	HBD1F	'5- CCCAGTTCCTGAAATCCTGA- 3'
10	HBD1R	'5- CAGGTGCCTTGAATTTTGGT- 3'
11	HBD2F	'5- CCAGCCATCAGCCATGAGGGT-3'
12	HBD2R	'5- GGAGCCCTTTCTGAATCCGCA-3'
13	M13F	5´-GTAAAACGACGGCCAG-3´
14	M13R	5´-CAGGAAACAGCTATGAC-3´

The tubes were inverted five times to mix and incubated on ice for 5 minutes. The tubes were centrifuged for 10 minutes at 1,000 g. The supernatant was transferred to a clean collection tube and 800 µl of PMR4 buffer was added to the supernatant. The mix was loaded to spin filter and centrifuged at 1,000 g for 30 seconds, and 500 µl of PMR5 buffer was added and centrifuged at 1,000 g for 30 seconds. The flowthrough was discarded and the spin filter was placed on a new collection tube, then 50 µl of PMR6 (RNase free water) was added to the center of the white filter membrane to elute RNA and centrifuged for 30 seconds.

Defensin genes DNA Amplification

From cows genomic DNA

DNA amplification was carried out using genomic DNA in a PCR reaction for 34 cycles under the following conditions: initial denaturation for 2min at 95 °C, denaturation at 95 °C for 1 min, annealing at 60 °C for 1min, extension at 72 °C for 1 min.

From human genomic DNA

Purified DNA was subjected to the PCR reaction under the following conditions: An initial denaturation step at 95 °C for 3 min, denaturataion at 94 °C for 1min, annealing at (57 °C, 60 °C and 62 °C) for 1 min, extention at 72 °C for 1min for 34 cycle followed by 10 min at 72 °C for final elongation.

Amplification of defensin gene from insect cDNA

cDNA synthesis

cDNA from insect total RNA was synthesized in 25 µl RT-PCR reaction containing 100 unit AMV reverse transcriptase (Promega, USA), 4 mM nucleotide mix, 10 pmole of primer, 10 ng of total RNA. Amplification was performed for 34 cycles of PCR with denaturation step at 94 °C for 30 seconds, annealing step at 55 °C for 30 seconds, extension step at 72 °C for 45 seconds and a finial extension step at 72 °C for 10 minutes. The PCR reaction products were analyzed by electrophoresis on 2% agarose.

PCR conditions

PCR reaction contained 1X Taq DNA, 4 mM $MgCl_2$, 1 U Go Taq Flexi DNA polymerase (Promega, USA), 4 mM nucleotide mix, 10 pmole of forward and reverse primers, 50 ng DNA in a 25 µl PCR reaction.

Recovery of DNA fragments from gel

DNA fragments were extracted from gel slices using EzWay Gel Extraction Kit. The desired band was excised from the agarose gel and transferred to 1.5 microcentrifuge tube containing extraction buffer. Samples were incubated at 50-55 °C for 5-10 min and tubes were inverted 2-3 times every 2 min until the gel slice is dissolved completely. The mixture was applied to the DNA column and centrifuged for 1 min. The flow- through was discarded the column was inserted in a collection tube. The column was washed with 750 µl of washing buffer and centrifuged for 1 min.

The column was transferred to a fresh 1.5ml microcentrifuge tube and 30-50 µl of elution buffer or sterile distilled water was added to the center of each column and let to stand for 1 min. DNA was eluted by centrifugation for 1 min.

Cloning of PCR products

The amplified PCR fragment was cleaned using PCR clean up kit (Maxim biotech INC, USA). The purified DNA fragments were then cloned in PCR - TOPO vector with TOPO TA cloning kit (Invitrogen, USA) and transformed into the competent *E. coli* strain TOPO 10. Plasmid DNA was isolated from 2 – 6 of selected white colonies and DNA was cut with restriction enzymes to test the insert orientation and confirmed for the right insert by PCR. Plasmid DNA was isolated with Qiagen miniprep column (Qiagen)

DNA Sequencing

PCR fragments were sequenced by (Macrogen, Korea). DNA sequences obtained from this study were deposited in the NCBI nucleotide sequence database (www.ncbi.nlm.nih.gov) and are listed (accession numbers).

RESULTS AND DISCUSSION

The main objective of this study is to obtain and isolate the defensin gene from various Egyptian organisms and draw the phylogenetic relationship among those molecules for the future use of them in biological control in the Egyptian environment. A total of 4 different species were chosen (insects, bovine, and human) as various sources of defensing genes. It is known that, small cationic peptides play an important role in host defense, as part of the innate immune system. These molecules are phylogenetically ancient and ubiquitous. They have been described in bacteria, moulds, plants, invertebrates and vertebrates, including mammals. The host defense peptides (HDPs) are evolutionarily active and have continued to respond to the varying challenges faced by mammalian species in their radiation over the past 100 million years. They have been working over a longer time-scale in animals and other organisms that lack adaptive immunity, where they play a primary defensive role (Sergio crovella, 2005).

Amplification of defensin genes from insect species

Total RNA was isolated from adult *Sitophilus oryzae* and *Callosobruchus chinensis* and used in RT-PCR. This resulted in the amplification of 150 bp amplicon (Figures, 1, 2). RT-PCR resulted in the obtaining of 150 nucleotides of defensin genes in Sitophilus oryzae (1) and Callosobruchus chinensis (Figure 2).

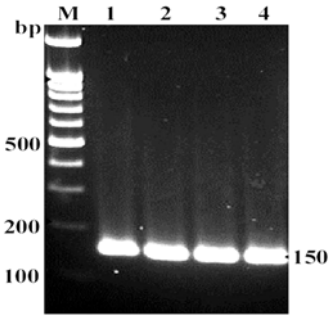

Figure 1: RT-PCR amplifications using RNA samples from adult insects *Sitophilus oryzae.* M: 100 bp DNA ladder, 1- 4 are four different insect samples.

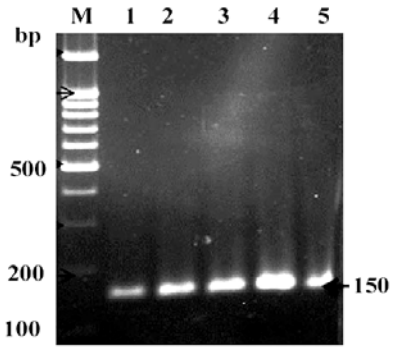

Figure 2: RT-PCR amplifications using RNA samples extracted from adult insects *C. chinensis.* M: 100 bp DNA ladder, 1-5: five different insect samples of *C. chinensis.*

Defensin-like peptides were reported in Lepidoptera (Mandrioli et al., 2003). Heliomicin peptide isolated from *Heliothis virescens* showed antifungal activity and shares similarities with antibacterial insect defensins (Lamberty et al., 1999). Mandrioli et al (2003) isolated a portion defensin gene by PCR amplification using two primers designed based on *M. brassicae* defensin gene sequence AF465486. Its expression was weak constitutive expression that was greatly increased by Gram positive, but not by Gram negative bacteria.

Amplification of defensin genes from native and Friesian cows (*Bos taurus)*

One amplicon of 429 bp was amplified from native cows, and three amplicons of 429 bp, 1300 bp, 1650 bp were amplified from Friesian cows (Figure 3, Figure 4).

The bovine linage was one of the first in which β- defensin- like molecule were discovered (Diamond and Bevings, 1998; Selsted et al. 1993). These previous studies resulted in 18 complete and partial bovine β-defensin (BBD) sequences being identified. Mammary gland of a cow is highly susceptible to inflammation. During infection with bacteria the number of polymorphonuclear leucocytes in milk increases dramatically (Hogan et al., 2001). Defensins can affect-the quality of cow milk. It was investigateed whether the defensin genotype affects the milk yield and quality in Black-and-White. Because of the antimicrobial role that defensins play in humans and animals, genes encoding these peptides may be considered as molecular markers of a genetically determined susceptibility or resistance of the mammary gland to mastitis. To determine the defensin gene polymorphism in Black and white cows, DNA was isolated from blood and the RFLP method with enzyme TaqI was used. Statistical evaluation included cows with more than seven records, and showing the 2.5 %frequency of combined defensin genotypes. Primers BBD-1S and BBD-2A were used for amplification of β- defensin gene fragment. Using these primers, the 1638 bp β1 defensin (enteric) gene fragment, encompassing parts of exon1 and 2 and the interveving intron was to be amplified. Two major amplicons of around 1650 bp and 1300 bp as well as several less abundant PCR products were amplified. This indicates that in addition to β1- defensin (enteric) the genes coding for other defensin were amplified. Reported sequences were submitted to the GenBank under the accession numbers EU599220 (B1) and EU599221 (B2).

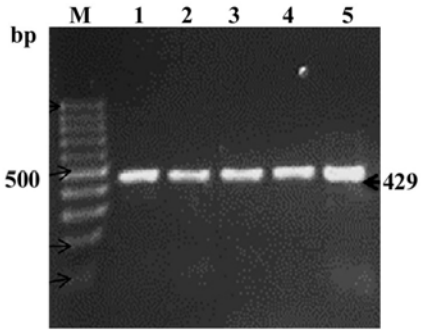

Figure 3: Agarose gel of PCR amplification products of beta-defensin gene from Egyptian cows. M: DNA ladder, 1-5 Egyptian cows samples.

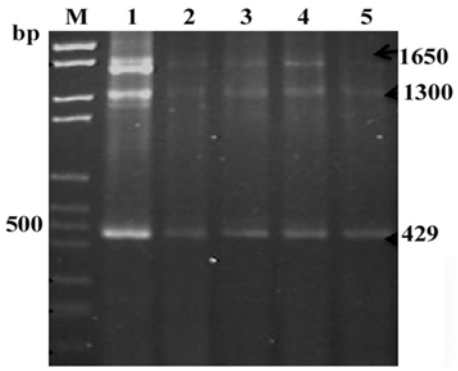

Figure 4: Agarose gel of PCR amplification products of beta-defensin gene from Friesian cows. M: DNA ladder, 1-5 Friesian cows samples.

The sequence alignment showed that B2 defensin gene (1650 bp) is closed to the other beta-defensin genes. Because of the similarity of sequence doesn't exceed more than 70%, we conclude that the B2 is a new gene added to the other beta- defensin genes. To demonstrate the similarity between B1 defensin gene (429 bp) and B2 defensin gene, sequence alignment was carried out using clustal W program version (1.8). The result showed that, high similarity was observed between the two examined defensin genes B1 and B2 (Figure 7). Moreover, B1 and B2 were found in one group with the isolated defensin genes *Bos Taurus* which had the same ancestor.

Amplification of defensin genes from human

Three human genomic DNA blood samples were used along with specific with HBD1, HBD2, HBD3, HBD3 primers (Figures, 4, 5). The results indicated that, with primer HBD1 there were three amplicons of 800 bp, 215 bp and 179 bp. With primer HBD2, there were two amplicons at position 1000 bp and 179 bp.

The expression of human β-defeinsins (hBDs) was investigated in the cornel epithelium. Alll epithelial tissue collected from cadaveric corneas expressed m.RNA for hBD-1. hBD-2. All expressed hBD-1 m.RNA showed product size 215 bp, while hBD-2 expressed only in two corneas from eight and its product was around 257 bp and five expressed hBD-3 m.RNA of 206 bp from eight corneas. The hBD-1 and hBD-3 provides a baseline defense to protect the cornea from infection. hBD-3 is the only human β-defensin that have antimicrobial activity and insensitive to salt concentration; therefore it may be particularly beneficial at the ocular surface where the salt content of tears may interfere with the activity of the other salt sensitive defensins (Alison et al., 2003). Novel induced gene- and protein expressed of HBD-2 was detected chronically infected kidneys in contrast to negative expression in normal non-infected renal tissue from tumor bearing kidneys. The indicated that some amplicons may have similar molecular weight for different species; therefore it must be found additional method to differentiate between them.

Alignment and Phylogenetic analysis

PCR products amplified with specific oligonucleotides for the defensin genes from different species were sequenced after their purification with PCR clean up kit and Gel extraction kit (Macrogen). The obtained sequences were aligned and a phylogenetic tree was established using CLUSTAL W2 (version 1.82). The phylogenetic tree was constructed by neighbour-joining method (Figure 7) based on the proportion of different amino acid sites, its reliability was assessed with boot strap replications using MEGA (version 4).

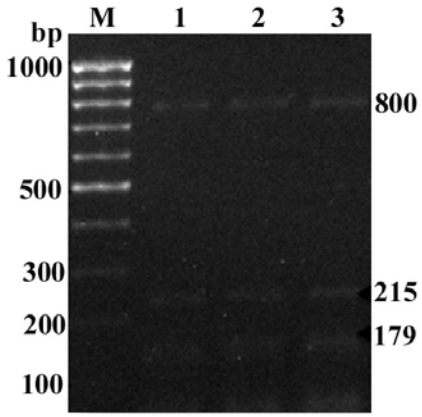

Figure 5: PCR amplification of human β-defensins 2. M: DNA marker; 1-3: Three different samples.

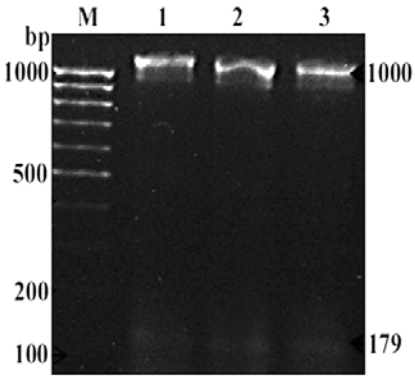

Figure 6: PCR amplification of human β-defensins 2. M: DNA marker; 1-3: Three different samples.

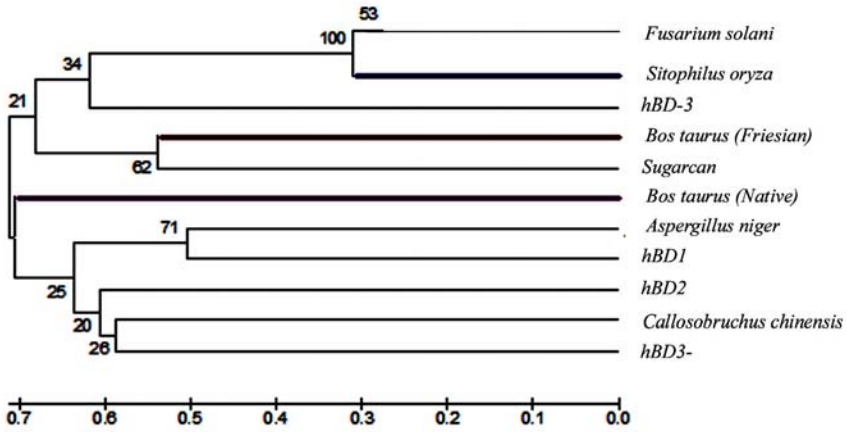

Figure 7: Phylogenetic tree of nucleotide sequences of different defensin genes.

Two clusters of genes are evident with human beta defensin genes1, 2, 3'- *C. chinensis* and Egyptian *Bos taurusi* in one subgroup supported by a 73% boot strap value. The other subgroup included Sugarcane, Friesian cow's hBD-3, *S. oryzae* and *F. solani* (Figure 7). The separation of the genes into sub-groups 1 and 2 also reflects the spatial arrangement of the genes. This distribution of defensins on the various locations of the phylogenetic tree improves the high difference among defensin within the same species as well as among different species.

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الملخص العربى

التعبير الجيني وعلاقة القرابة لجينات المناعة من مصادر مصرية للتحكم الحيوي

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تمثل ببتيدات الدفينسين أحد مجاميع الببتيدات المضادة للميكروبات والتي تلعب دورا أساسيا في المناعة الغير مستحثة في معظم الكائنات حقيقية النواة من الحشرات حتي الثدييات، وهي تمثل مدي واسع من التركيب وميكانيكية العمل معتمدة علي مصدرها. الهدف الرئيسي من هذه الدراسة هو عزل جين ببتيدات الدفينسين من مصادر كائنات حية من البيئة المصرية ودراسة علاقة القرابة الوراثية الفيلوجينية بين هذه الكائنات بواسطة هذه التتابعات. وفي هذه الدراسة، تم عزل تتابع جين الدفينسين من الكائنات التالية *Sitophilus oryzae* (rice weevil)، *Callosobruchus chinensis*، *Bos Taurus* (Egyptian and Friesian)، *Homo sapiens* (human blood cells) بإستخدام تقنية تفاعل البلمرة المتسلسل PCR، وتم كذلك قراءة التتابع النيكليوتيدي للجينات التي تم عزلها. تم عزل تتابع قدرة ١٥٠ نيكليوتيدة من كل من *Sitophilus oryzae* (rice weevil)، *Callosobruchus chinensis*، وتتابع قدره ٤٢٩ نيكليوتيدة من الأبقار المصرية Egyptiam cows، وثلاثة تتابعات ٤٢٩، ١٣٠٠، ١٦٥٠ نيكليوتيدة من أبقار الفريزيان Friesian cows. تم كذلك عزل ثلاثة تتابعات بإستخدام بادئات جين الدفينسين human β-defensin-1 وهي ١٧٩، ٢١٥، ٨٠٠ نيكليوتيدة، وتتابعين بإستخدام بادئات جين الدفينسين human β-defensin-2 وهي ١٧٩، ١٠٠٠ نيكليوتيدة. تم إستخدام تتابعات الجينات المعزولة لبناء شجرة القرابة الوراثية الفيلوجينية بين الأنواع المستخدمة في هذه الدراسة، والتي أكدت أن جينات الدفينسين المعزولة من الكائنات الحية من البيئة المصرية مختلفة إختلافا كبيرا في تتابعاتها وذلك داخل النوع الواحد أو بين الأنواع المختلفة. نتائج هذه الدراسة تعتبر خطوة أساسية لعزل مضادات الميكروبات من الكائنات الحية المصرية وإستخدامها كمضادات حيوية تجارية.

الكلمات الدليلية: ديفنسين، مادة وراثية، PCR