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An anionic defensin from *Plutella xylostella* with potential activity against *Bacillus thuringiensis*

X.-X. Xu[1]†, Y.-Q. Zhang[1]†, S. Freed[2], J. Yu[1], Y.-F. Gao[1], S. Wang[1], L.-N. Ouyang[1], W.-Y. Ju[1] and F.-L. Jin[1]*

[1]Key Laboratory of Bio-Pesticide Innovation and Application of Guangdong Province, College of Agriculture, South China Agricultural University, Guangzhou 510642, P. R. China: [2]Department of Entomology, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan 60800, Pakistan

Abstract

Insect defensins, are cationic peptides that play an important role in immunity against microbial infection. In the present study, an anionic defensin from *Plutella xylostella*, (designated as Px*Def*) was first cloned and characterized. Amino acid sequence analysis showed that the mature peptide owned characteristic six-cysteine motifs with predicted isoelectric point of 5.57, indicating an anionic defensin. Quantitative real-time polymerase chain reaction analysis showed that Px*Def* was significantly induced in epidermis, fat body, midgut and hemocytes after injection of heat-inactivated *Bacillus thuringiensis*, while such an induction was delayed by the injection of live *B. thuringiensis* in the 4th instar larvae of *P. xylostella*. Knocking down the expression of nuclear transcription factor Dorsal in *P. xylostella* by RNA interference significantly decreased the mRNA level of Px*Def*, and increased the sensitivity of *P. xylostella* larvae to the infection by live *B. thuringiensis*. The purified recombinant mature peptide (PxDef) showed higher activity against Gram-positive bacteria, with the minimum inhibition concentrations of 1.6 and 2.6 μM against *B. thuringiensis* and *Bacillus subtilis*, respectively. To our knowledge, this is the first report about an anionic Px*Def*, which may play an important role in the immune system of *P. xylostella* against *B. thuringiensis*.

Keywords: Antimicrobial activity, *Bacillus thuringiensis*, defensin, expression, *Plutella xylostella*, RNAi

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Introduction

Every creature in the biological world has a set of effective survival defense mechanisms to adapt the environment (Boman, 1995). Vertebrates have acquired immune system, which relies on B and T lymphocytes to resist the infection of pathogenic microorganisms. On contrary to this, invertebrates like insects lack the acquired immune system and solely rely on the innate immune system comprised of cellular and humoral immunity to produce antimicrobial effectors to resist pathogenic microorganisms (Ferrandon *et al.*, 2007; Lemaitre & Hoffmann, 2007; Vallet *et al.*, 2008; Bao *et al.*, 2014).

Antimicrobial peptides (AMPs) are small polypeptides of 20–60 amino acid residues with molecular sizes of 2–7 kDa (Pütsep *et al.*, 1999). These active peptides are alkaline and heat stable with a broad spectrum of antibacterial activity. Known insect AMPs are classified into three major groups based on their structures. Linear AMPs with α-helical conformation but without cysteine; ring-type AMPs with cysteine

*Address for correspondence
Phone: +86 2085280203
Fax: +86 20 85280293
E-mail: jflbang@scau.edu.cn
†These authors contributed equally to this work.

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and AMPs have high contents of one or two particular amino acids, most frequently proline and/or glycine residues (Meister *et al.*, 1997; Bulet & Stöcklin, 2005; Jin *et al.*, 2012). The expression of all the AMPs is activated by microbial infection and regulated by two distinct intracellular signal transduction pathways i.e., the immune deficiency (IMD) and Toll pathways, which also regulate the transcription of hundreds of other genes by controlling the nuclear translocation of the NF-κB transcription factors Relish, Dorsal and Dorsal-related immunity factor (DIF) (Gobert, 2003; Hultmark, 2003; Ferrandon *et al.*, 2007). Dorsal and DIF are activated by the Toll pathway in response to infection with fungi and Gram-positive bacteria. These factors are localized in the cytoplasm and interact with the mammalian IκB homologue Cactus in unstimulated cells. In response to an infection, DIF and Dorsal translocate into the nucleus by dissociating from Cactus and activate antimicrobial peptide genes to express and release into the hemocoel to defend the hosts against the infection (Lemaitre *et al.*, 1997).

Insect defensins, are in general a family of cationic and inducible AMPs that bear six cysteine residues engaged in three intra-molecular disulfide bridges. These are more active against Gram-positive bacteria and less against Gram-negative bacteria and fungi (Dang *et al.*, 2010). Defensins have been found in a number of insect orders including Coleoptera (Bulet *et al.*, 1991; Xu & Faisal, 2010; Nakamura *et al.*, 2011; Yi *et al.*, 2014), Diptera (Richman *et al.*, 1996; Dassanayake *et al.*, 2006; Dixit *et al.*, 2008; Dang *et al.*, 2010) and Lepidoptera (Kaneko *et al.*, 2008) and insect defensin like molecule from mussels and scorpions. In addition to cationic defensins in insects, anionic defensins from *Bombyx mori* have also been reported (Wen *et al.*, 2009; Song *et al.*, 2010), which indicate their structural dissimilarities. The first two insect defensins were isolated from culture cells of the *Sarcophaga peregrina* (Matsuyama & Natori, 1988) and by and collaborators from bacteria-challenged larvae of the *Phormia terranovae* (Lambert *et al.*, 1989). Members of the defensins share a general feature and structure, while they correspond to molecular diversity in amino acid composition and size. The peptide sequences are relatively alike in an insect order but very much different across the orders.

The diamondback moth, *Plutella xylostella*, is a worldwide pest of cruciferous crops. With the abuse of pesticides, *P. xylostella* has developed resistance to most of the conventional pesticides and *B. thuringiensis* in the fields (Tabashnik, 1994; Pardo-Lopez & Soberon Bravo, 2013). There is growing recognition that an important element of gut microbiota of both invertebrates and vertebrates is their ability to change the host immune response. In certain conditions this effect can become lethal to the insects. For instance, activation of the immune responses by the bacteria leads to chronic conditions (Bates *et al.*, 2007; Nenci *et al.*, 2007; Maillet *et al.*, 2008). Current studies have also linked activation of the immune response of Lepidoptera to ingestion of non-lethal doses of *B. thuringiensis*. The ingestion of *B. thuringiensis* by greater wax moth, *Galleria mellonella* larvae increased both oxidative stress in the gut (Dubovskiy *et al.*, 2008*a*) and the phagocytic activity of hemocytes (Dubovskiy *et al.*, 2008*b*), while the exposure of *Bt* reduced the hemocytes and components of the humoral immune system in cabbage lopper *Trichoplusia ni* (Ericsson *et al.*, 2009). Insect defensins, a significant group of gene-encoded effectors of innate immunity play a vital part in resisting to the infections caused by different biological agents including bacteria and fungi (Ferrandon *et al.*, 2007; Lemaitre & Hoffmann, 2007; Dang *et al.*, 2010; Tamura *et al.*, 2011). Up to now, more than 60 defensins have been identified from different species of insect orders including Lepidoptera (Yi *et al.*, 2014; Badapanda & Surendra, 2016). The previous reports depict that majority of the defensins are isolated from the hemolymph, fat body or midgut of bacteria-challenged larvae (Lambert *et al.*, 1989; Boulanger *et al.*, 2004; Seufi *et al.*, 2011; Cecilia *et al.*, 2014). Insect defensins are classified according to their activity, while the antimicrobial defensins are recognized to be active mainly against Gram-positive bacteria at different concentrations (Bjellqvist *et al.*, *1993*; Chenna *et al.*, *2003*; Boulanger *et al.*, *2004*; Yang et al., *2009*; Bhusan *et al.*, *2010*; Seufi *et al.*, 2011; Wang *et al.*, *2012*; Telleria *et al.*, *2013*). In the current work, we firstly report the immunological responses of defensins of *P. xylostella* after challenging with *B. thuringiensis*. With the analysis of transcriptome sequence of *P. xylostella* we found only one unique sequence encoding defensin, which has high similarity to the defensin sequence from the *P. xylostella* genomic sequence and owned the characterization of anionic peptide. In order to elucidate its functions against pathogenic bacterium *B. thuringiensis*, in the present study, the cloning and characterization of the novel anionic defensin from *P. xylostella* were reported. The spatial tissue distribution and temporal expression after infection of *B. thuringiensis*, as well as the antimicrobial activity were also described. In addition, its expression regulated by the Toll-Dorsal pathway as analyzed by RNAi. Our findings may help shed light on immune responses of *P. xylostella* against *B. thuringiensis* and have implications for using pathogenic bacteria for the biological control of pests in practice.

Materials and methods

Insect rearing, microorganisms and immunization

The larvae of *P. xylostella* were collected from the cabbage fields and reared successively on cabbage in the greenhouse of Engineering Research Center of Biological Control, South China Agricultural University, China, at 25 ± 2°C, 70% relative humidity (RH) and a photoperiod of 16:8 h (light : dark).

Gram-negative bacteria, i.e. *Escherichia coli* DH5a, BL21 (DE3), *Bacillus cereus, Bacillus subtilis, Bacillus pumilus*; Gram-positive bacteria, i.e. *Pseudomonas fluorescent, Salmonella choleraesuis, Staphylococcus aureus* Rosenbach; and fungi *Botrytis cinerea* Klotzsch, *Penicillium crustosum* Thom, *Peronophythora litchi, Colletotrichum orbicular* Berk, *Fusarium oxysporum* Schlecht, *Colletotrichum gloeosporioiees* Penz. were obtained from the Research Institute of Microbiology, Guangzhou, China, while the insect pathogenic fungus, while the insect pathogenic fungus, *M. anisopliae* (MaQ 10) was provided by Dr Qiongbo Hu (South China Agricultural University, Guangzhou, China), which was kept in China Center for Type Culture Collection (No. CCTCCM 208173). *B. thuringiensis* (HD73) was kindly provided by Prof. Jie Zhang (The Chinese Academy of Agricultural Sciences, Institute of Plant Protection, Plant Diseases and Insect Pests, Beijing, China).

The eggs, 1st–4th instar larvae, prepupae, pupae and adults were collected and used in fresh. Each 4th instar larva was injected separately with log phase heat-inactivated *B. thuringiensis* (2×10^6 CFU ml^{-1}) or live *B. thuringiensis* (2×10^6 CFU ml^{-1}) in the abdominal region. Every 6 h after injection with *B. thuringiensis* until 48 h post injection, the fat body, midgut, and epidermis were dissected and collected in ice-cold tubes (Xu *et al.*, 2007). The hemolymph from the *Bt* injected larvae was collected into 1/10 volume of anticoagulant

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Table 1. Primers used for cloning, expression and RNAi analysis.

Primer name	Sequence	Information
Px*Def*dF1	5′- ACTGCTTACTCGAAGACCCTCA-3′	RT-PCR
Px*Def* dF1	5′- ATTTCACCAAGTTATTGTTCGTTA-3′	RT-PCR
Px*Def* dR2	5′- ACCGGTAGCTGACACAAATACCGTA-3′	RT-PCR
Px*Def*3	5′-GCAGGAGACAGTGGTTGAGGAG-3′	3′RACE
Px*Def*5	5′- CAGTCCTCTGCACCGCCACATCTCTCGGG -3′	5′RACE
UMP-S	5′-CTAATACGAC TCACTATAGGGC-3′	RACE
Def-qF	5′-CCAACCGGTCAACAGTCAAAATG -3′	qRT-PCR
Def-qR	5′-TCTCGGGTAACACAAAGCACTCG-3′	qRT-PCR
β-acF	5′-TGGCACCACACCTTCTAC-3′	Internal control
β-acR	5′-CATGATCTGGGTCATCTTCT-3′	Internal control
DefEF	5′-GCGCCATG**G**GAATACCTTGCCAGTACGAAGACG -3′	Expression
DefER	5′-CCTCGAGTCAAACACAGCTGCACCGGTAGCTGACAC-3′	Expression
GFP-F	5′*GATCCTAATACGACTCACTATAGG*AAGGGCGAGGAGCTGTTCACCG-3′	GFP-dsRNA
GFP-R	5′*GGATCCTAATACGACTCACTATAGG*CAGCAGGACCATGTGATCGCGC-3′	GFP-dsRNA
Def-RNAiF	5′*GGATCCTAATACGACTCACTATAGG*TTCTACAGTCCAACCGGTCAACA-3′	Def-dsRNA
Def-RNAiR	5′*GGATCCTAATACGACTCACTATAGG*TATTGTCACTGAGAATCAAACAC-3′	Def-dsRNA
Dor-RNAiF	5′*GGATCCTAATACGACTCACTATAGG*CTGAAGCGAAAGCGACAGGAAACC-3′	Dor-dsRNA
Dor-RNAiR	5′*GGATCCTAATACGACTCACTATAGG*CTGCTGCGCCATAGGAGCCATA-3′	Dor-dsRNA

Primer DefEF, Bold/underlined letter of G represents a designated mutation of letter A to G.
Sequence in Italic (*GGATCCTAATACGACTCACTATA*) represents T7 promoter.

buffer (10% sodium citrate, pH 7.0, and 200 mM phenylthiourea) from the abdominal prolegs of the normal and immune-challenged larvae at 24 h post-injection. The hemocytes were collected by centrifugation at 3000 × g for 10 min at 4°C.

Cloning of defensin from P. xylostella

The RNA was extracted from the above dissected tissues using Trizol reagent and was reverse-transcribed into first-strand cDNA using SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A UniGene sequence encoding defensin was obtained from *P. xylostella* transcriptome and was validated by polymerase chain reaction (PCR) using specific primers (table 1).

Gene specific primers (table 1) were designed based on the sequence of the cloned cDNA fragment to amplify cDNA by using SMART™ RACE cDNA amplification kit (Clontech, USA) following the manufacturer's instructions. PCRs were carried out by using the Advantage 2 PCR kit (Clontech, USA) according to the following conditions: 3 min at 94°C, 5 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 30 s; 3 min at 94°C, 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and a 8 min elongation at 72°C. The resultant PCR products were subjected to electrophoresis on 1.0% agarose gel to determine the band sizes. The expected sizes bands were excised and purified using an OMEGA gel purification kit (OMEGA, USA). The purified cDNA fragments were cloned into the pMD-18T vector (TaKaRa, Japan) according to the manufacturer's instructions and sequenced. The complete cDNA sequence encoding Px*Def* was obtained by overlapping the PCR.

*Sequence analysis of Px*Def

The software Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to analyze the ORF of Px*Def*. The similarity index was analyzed by BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast), while, multiple sequence alignment was carried out by ClustalW (http://www.ebi.ac.ek/clustalw). The signal peptide was analyzed by Signal P4.1 server (http://www.cbs.dtu.dk/services/SignalP/), while, the trans-membrane region was predicted by TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/). The molecular weight and isoelectric point (pI) were determined by using ScanProsite (http://prosite.expasy.org/scanprosite/). MEGA 5.2 software was used to construct the phylogenetic tree on the basis of the Neighbor Joining method (NJ) to assess the relationship of the defensin sequences (Tamura *et al.*, 2011).

Quantitative real-time PCR (qRT-PCR) analysis

The transcription level of Px*Def* in the developmental stages and dissected tissues after bacterial challenge was measured by qRT-PCR using a CFX96 real-time system. Approximately 1 µg RNA from each sample was reverse-transcribed using the PrimeScript^{RT} Reagent Kit with a DNA Eraser (TaKaRa, Japan). The first-strand cDNA was subsequently used as a template for PCR with the Def-qF and Def-qR primers (table 1), while β-actin (GenBank accession No. AB282645) was used as the internal control for cDNA normalization and amplification by using the β-actinF and β-actinR primers. The qRT-PCR mixture consisted of 2 µl cDNA sample, 8.5 µl ddH_2O, 12.5 µl SYBR Premix Ex Taq™ (TaKaRa, Japan) and 1 µl of each gene-specific primer (10 µM). The PCR conditions consisted of: 95°C for 30 s; 40 cycles of 95°C for 10 s, 58°C for 30 s and 72°C for 20 s, followed by dissociation curve analysis at 95°C for 10 s, 65°C for 5 s and 95°C for 15 s to verify the amplification of a single product. The threshold cycle (CT) values were determined by CFX96 software. The data were exported into a Microsoft Excel spread sheet for subsequent analysis, where the relative expression ratios of the target gene in treated and untreated control groups were calculated using a $2^{-\Delta\Delta CT}$ method. All assays were performed in three independent replicates.

*Recombinant expression and purification of Px*Def

The cDNA fragment encoding the mature peptide of PxDef (RIPCQYEDATEDTICQQHCLPKGYSYGICVSYRCSCV) was obtained by RT-PCR with DefEF/DefER primers. In order to

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obtain mature PxDef with the native N-terminus, the *Nco* I restriction enzyme was introduced at the cleavage site on the N-terminus of the Px*Def* nucleotide sequence and mutated the A to G in the primer of DefEF. The products of RT-PCR were purified using agarose gel electrophoresis, digested with *Nco* I and *Xho* I enzymes, and then ligated into the *Nco* I-/*Xho* I digested expression vector pET-32a(+) (Novagen, USA). The constructed plasmid was transformed into competent BL21 (DE3) cells for expression of the TRX (thioredoxin)-6His-PxDef-fusion proteins. The fusion protein was expressed in BL21 (DE3) cells after induction with 0.5 mM isopropyl-D-thiogalactoside (IPTG) for 6 h at 28°C. The bacterial pellets were harvested by centrifugation and lysed with lysis solution (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5% Triton X-100, 2 mg ml^{-1} lysozyme). After sonication, the supernatants were recovered by centrifugation and subjected to Ni^{2+}-NTA chromatography for purification of the recombinant fusion protein, which was dialyzed and dissolved in PBS (pH 7.4) to a final concentration of 1 mg ml^{-1}. In order to release Px*Def* from fusion proteins, enterokinase was added at a concentration of 8 U mg^{-1} of protein, and the digestion was performed with the reaction buffer (150 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 2.5 mM $CaCl_2$) at 21°C for 16 h with stirring according to the manufacturer's protocol. The reaction mixture was again subjected to HisTrap HP column to remove thioredoxin and the undigested fusion proteins. The flow-through fractions containing PxDef was collected, concentrated with PEG2000, dialyzed in double distilled water for desalination, and finally lyophilized. The purified recombinant protein PxDef was analyzed by Tricine-SDS–PAGE (Jin *et al.*, 2012).

Antibacterial assay

The activity of recombinant protein PxDef against *B. thuringiensis* was checked by agar diffusion method as described previously (Xu *et al.*, 2007). The minimal growth inhibition concentration (MIC) was determined by liquid growth inhibition assay (Kennerdell & Carthew, 1998) and expressed as the lowest final concentration of the peptide at which no growth was observed (Dhume *et al.*, 2006). Five Gram-positive bacteria, three Gram-negative bacteria and seven fungi were used to test the antimicrobial activity of the purified PxDef. The purified PxDef was serially diluted 10-fold with ultrapure water and aliquots (10 µl) from each dilution were distributed to a 96-well polypropylene microtiter plate, and each well was inoculated with 100 µl mid-log phase bacterial suspension (1×10^5 CFU ml^{-1}) in LB broth.

RNA interference in P. xylostella

Double stranded RNAs (dsRNAs) specific to Px*Def* and Px*Dor* (GenBank accession number: KF960046) were prepared with gene-specific primers by RT-PCR using T7 RiboMAX™ Express RNAi System (Promega, USA) according to the manufacturer's protocol. The primers (table 1) were designed using the SnapDragon dsRNA design software (http://www.flyrnai.org/cgi-bin/RNAi-find-primers.pl). To minimize potential off-target effects, a dsRNA segment corresponding to the green fluorescent protein (GFP) was also synthesized as a negative control as described previously (Huang *et al.*, 2015). PCR reactions (20 µl) were conducted at an initial denaturation of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and an extension at 72°C for 30 s. The PCR products were purified following the manufacturer's instructions for dsRNA synthesis. The reaction mixture was incubated at 42°C for 16 h, followed by 75°C for 5 min, and 25°C for 30 min. The dsRNA was precipitated with 5 M ammonium acetate and isopropanol, resuspended in ultrapure water and quantified by a spectrophotometer at wave length of 260 nm (Nanodrop 2000, USA).

The 4th instar *P. xylostella* larvae were injected with 1 µl dsRNA to Dorsal (1 µg $µl^{-1}$, forty larvae), 1 µl dsRNA to defensin (1 µg $µl^{-1}$, forty larvae) and 1 µl dsRNA to GFP (1 µg $µl^{-1}$, forty larvae) and 1 µl DEPC-H_2O (1 µl/larvae, forty larvae), respectively. While dsGFP was taken as non-protein injection control, DEPC-H_2O was taken as injection control and CK was taken as no-injection control. After 24 h of RNAi treatment, in order to stimulate the immune response, the all 200 larvae were fed on live *B. thuringiensis* (HD73) by leaf-dipping method (Tabashnik & Cushing, 1987). The control larvae were treated with phosphate-saline buffer (PBS, pH 7.2). After every 12 h, fat body was extracted from bacterial-challenged larvae and qRT-PCR was performed to analyze the expression of *P. xylostella* defensin. Each treatment was performed in three independent replicates.

Bioassay on B. thuringiensis *pathogenicity*

The 4th instar larvae were treated with dsRNA specific to Px*Def* as described above. After 24 h, the larvae were fed on live *B. thuringiensis* treated cabbage leaves. While the control larvae were treated with PBS (pH 7.2) only. Each treatment was performed in three independent replicates.

Statistical analysis

The figures were made with the SYSTAT SigmaPlot 12, Excel 2010 and SPSS19 software with one representative set of data. One-way ANOVA tests were performed using SPSS 19.0 to determine significant differences between the tested and control groups at different time in the same tissue ($P \leq 0.001$).

Results

*cDNA cloning and sequence analysis of Px*Def

A 342 bp cDNA fragment was obtained from the transcriptome (Lin *et al.*, 2013) and validated by RT-PCR. A complete cDNA sequence encoding defensin (Px*Def*) was obtained by overlapping the RACE cDNA fragments (GenBank No. KF960050) (fig. S1). The deduced amino acid sequence of Px*Def* was 96% identical to the defensin from *P. xylostella* Genome (You *et al.*, 2013), indicating that Px*Def* is the transcription product from *P. xylostella* Genome (fig. S2).The phylogenetic analysis showed that Px*Def* is significantly similar to defensins from other Lepidopteran insects (fig. S3). Sequence analysis showed the presence of potential signal sequence of 22 residues, a propeptide of 60 residues and a mature active peptide of 37 residues with a molecular mass of 4.2 kDa (fig. S1). The mature peptide contains characteristic six-cysteine motif of insect defensins, with a predicted isoelectric point of 5.57, indicated that it is an anionic defensin. In addition, a transmembrane region of 6 to 29 amino acid residues can form an alpha helix, which is consistent with previous reports (Gao & Zhu, 2013; Lee *et al.*, 2013). A special fragment (Pro64-Pro79) was found in Px*Def* propeptide, which is longer than that in defensins from other Lepidopteran insect species (fig. S4).

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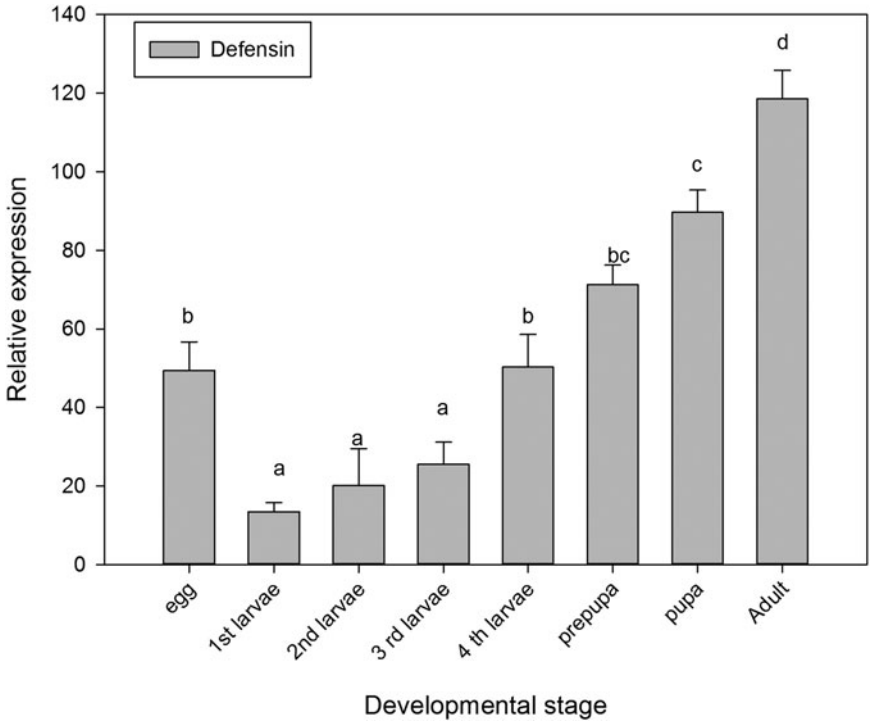


Fig. 1. Developmental expression of *PxDef* in *P. xylostella* from eggs to adults. The developmental expression of *PxDef* transcript in *P. xylostella* was determined by real-time quantitative RT-PCR. Expression of *PxDef* mRNA is relative to β-actin mRNA. Comparison of *PxDef* expression levels at different developmental stages was performed by *t*-test. Data with different letters are significantly different ($P < 0.05$) (DMRT) among different developmental stages.

*Expression patterns of Px*Def

qRT-PCR analysis showed the expression of Px*Def* in all developmental stages, from eggs to adults. In comparison, the mRNA level in pupae and adults was significantly higher than in the larval stages (fig. 1).

The 4th instar larvae of *P. xylostella* injected with *B. thuringiensis* were analyzed for the expression of Px*Def* in epidermis, hemocytes, midgut, and fat body. Px*Def* was significantly induced in all four tissues after injection of heat-inactivated *B. thuringiensis*. Despite the differences in the expression, highest levels of expression were found at 24 h in hemocytes and epidermis, 30 h in midgut, and 36 h in fat body (fig. 2). After injection of live *B. thuringiensis*, the expression of Px*Def* was significantly decreased in epidermis, midgut and hemocytes up to 30 h post injection and in the fat boy until 36 h post-injection, then increased rapidly. The results suggest that live *B. thuringiensis* may release some effectors to inhibit the expression of Px*Def* (fig. 2).

RNA interference (RNAi)

To elucidate the function of Px*Def* in immunity, DEPC, GFP-dsRNA, Px*Def*-dsRNA and Px*Dor*-dsRNA were injected into 4th instar larvae of *P. xylostella* separately. Injection of dsRNA to *PxDef* significantly down regulated expression of Px*Def* mRNA at 12 h post- injection, and the RNAi effect sustained until 48 h post-injection (fig 3). Furthermore, RNAi silencing of Px*Dor* also significantly down regulated Px*Def* transcript from 12 to 48 h post-injection (fig. 3), suggesting that expression of Px*Def* is regulated by the Toll/Dorsal pathway.

After RNAi silencing of Px*Def* and Px*Dor* separately, the susceptibility of larval feeding by live *B. thuringiensis* (2×10^6 CFU ml^{-1}) was analyzed (fig. 4). Control larvae (naïve and GFP-dsRNA injected larvae) showed approximately 20–30% mortality, while larvae treated with dsRNAs to Px*Dor* and Px*Def* showed significantly higher mortality than the control larvae 3 days after the infection of *B. thuringiensis*. The results suggest that PxDef may play a role in defense against *B. thuringiensis* infection in *P. xylostella*.

*Antibacterial activity of recombinant Px*Def

Recombinant PxDef was expressed as a fusion protein to thioredoxin (Ando *et al.*, 1987). Recombinant PxDef fusion protein was purified by affinity chromatography, thioredoxin was removed by enterokinase and recombinant PxDef was further purified. Analysis of the purified recombinant PxDef by Tricine-SDS–PAGE revealed that PxDef was purified to homogeneity with a molecular mass of approximately 4.2 kDa (fig. 5a).

In order to test antimicrobial activity of recombinant PxDef against microorganisms, serial dilutions of purified recombinant PxDef was incubated with bacterial and fungal cultures, and the minimal inhibitory concentrations (MICs) were determined. Recombinant PxDef showed high activity against all Gram-positive bacteria and two fungi (*Metarhizium anisopliae* and *Fusarium oxysporum*) tested, showed relatively high activity against other fungi, but low activity against all Gram-negative bacteria tested (table 2).

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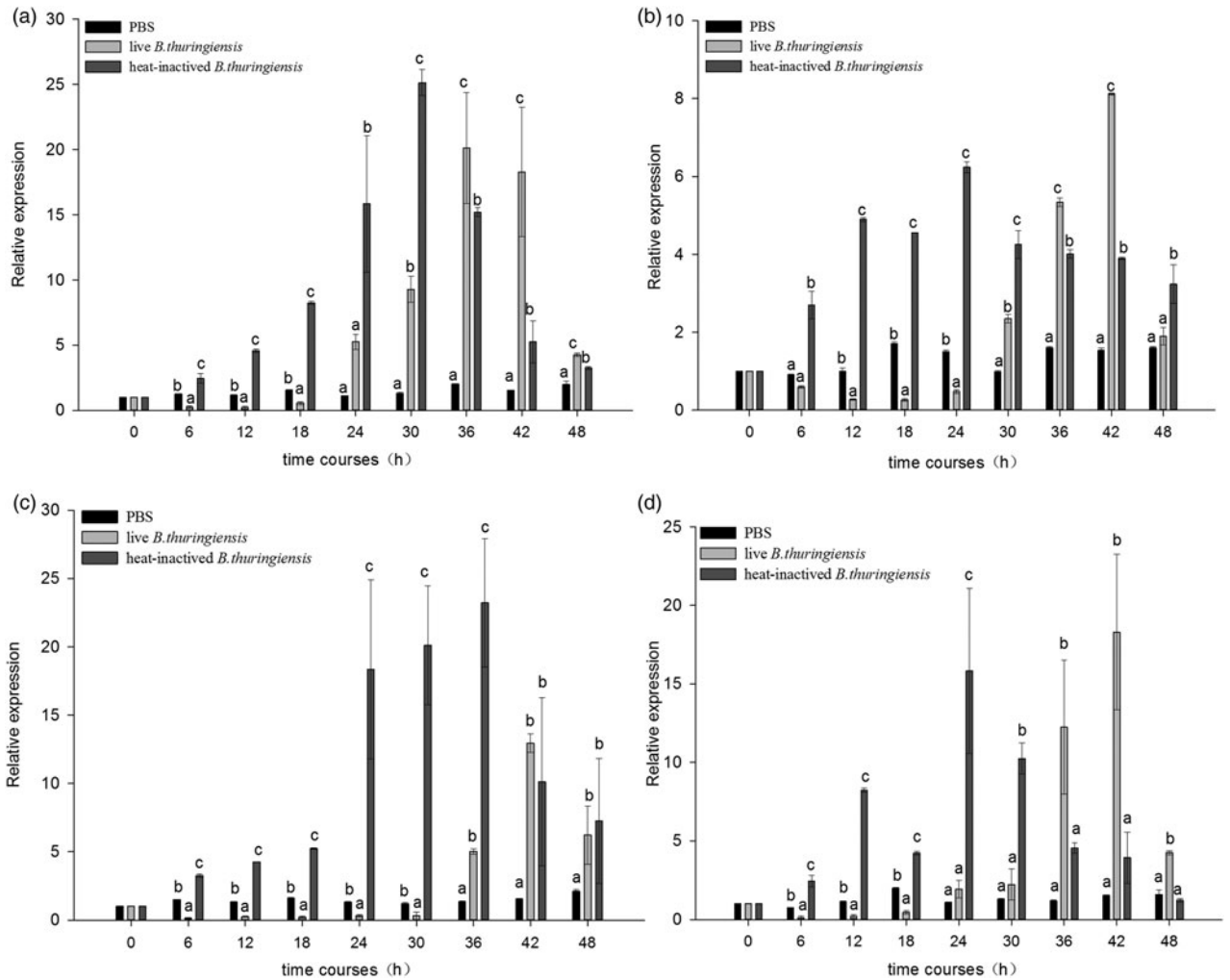


Fig. 2. Induced expression of Px*Def* in different tissues after *B. thuringiensis* challenge. The mRNA level of Px*Def* in midgut (a), epidermis (b), fat body (c), and hemocytes (d) was analyzed by qRT-PCR. Comparison of Px*Def* mRNA levels was performed by *t*-test. Data with different letters are significantly different ($P < 0.05$) (DMRT) among time courses and tissue distribution.

Recombinant PxDef antibacterial against *B. thuringiensis* was performed. The inhibition zone was quantified by measuring distance from the edge of the paper disk to the edge of the clear zone (fig. 5b). The results showed that recombinant PxDef at 0.5 µg displayed activity against *B. thuringiensis* with inhibition zone of approximately 0.5 cm compared with inhibition zone of ∼0.7 cm by ampicillin (0.5 µg).

Discussion

Insect defensins, the largest group of insect AMPs, have high activity against Gram-positive bacteria and lower activity against Gram-negative bacteria and fungi (Dang *et al.*, 2010). To date, most defensins isolated and identified in insects are cationic peptides (Dimarcq *et al.*, 1994; Chernysh *et al.*, 1996; Lauth *et al.*, 1998; Lee *et al.*, 2004; Seufi *et al.*, 2011; El Shazely *et al.*, 2013; Porto *et al.*, 2014), but little is known about anionic peptides in Lepidopteran insects, especially in *P. xylostella*. In the present study, Px*Def* cDNA clone was isolated and sequenced, and the deduced amino acid sequence comprised six cysteine residues in the conserved positions that are typical structural characteristics of invertebrate defensins. Multiple sequence alignment showed that there is a special fragment (Pro64-Pro79) Px*Def*, which is longer than that in other defensins from Lepidoptera (fig. S4). The special amino acid sequence suggests that Px*Def* may be different from most defensins reported so far. It is also possible that the longer fragment may account for the anionic pI of Px*Def*.

Defensins are reported to be cysteine-rich cationic AMPs. In our study, the pI value of mature Px*Def* is 5.57, indicating that it is an anionic peptide, different to almost all of the known defensins. Recently, an anionic defensin-like gene from silkworm (Kaneko *et al.*, 2008) and defensin from the tick *Amblyomma hebraeum* (Lai *et al.*, 2004) have been reported. *A. hebraeum* defensin (AhDef) contains a net negative charge with a pI value of 4.44, and it exerts antimicrobial activity against Gram-negative bacterium *E. coli* and Gram-positive bacterium *S. aureus* (Lai *et al.*, 2004). However, in the present study, recombinant PxDef exhibited higher activity against Gram-positive bacteria and fungi, but lower activity against *E. coli* and other Gram-negative bacteria. The antibacterial mechanism of AMPs is ascribed to the interaction between

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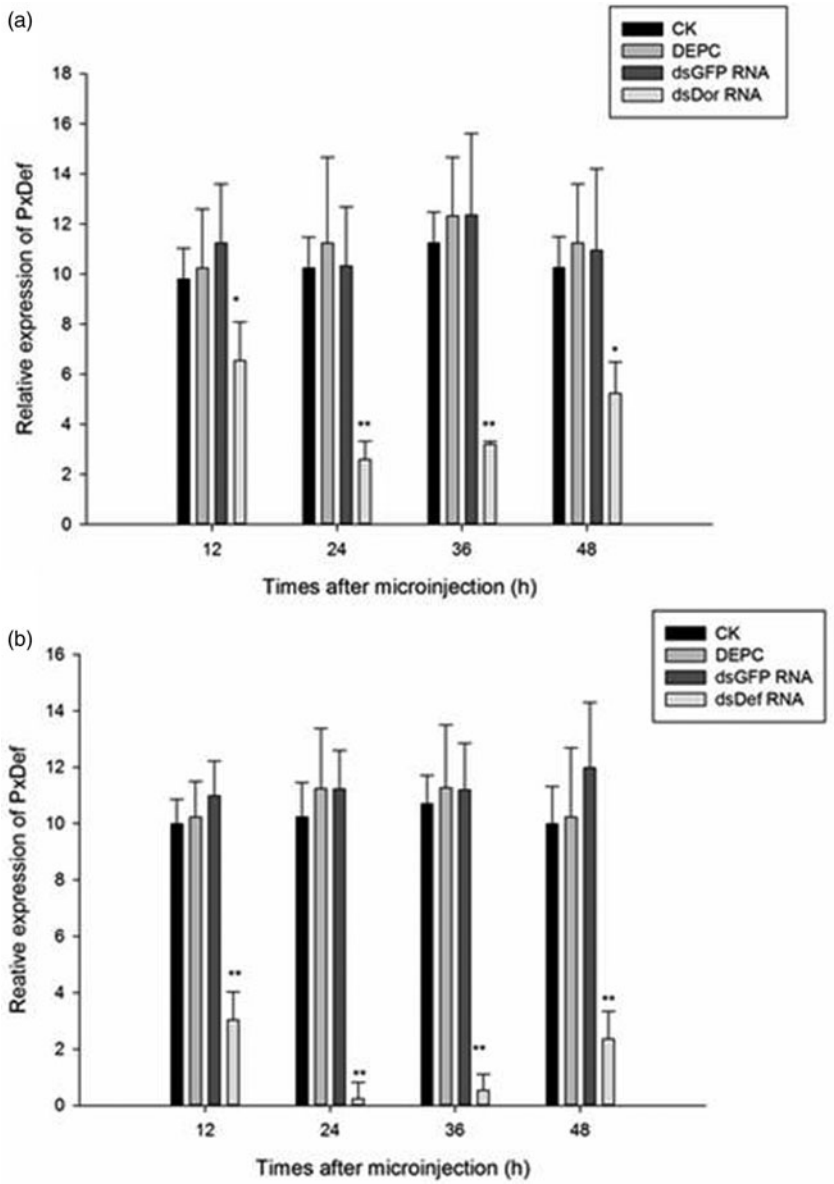


Fig. 3. RNAi silencing of Px*Dor* (a) and PxDef (b). *P. xylostella* larvae were injected with DEPC-treated water (CK), dsGFP RNA, dsPxDor RNA, or dsPxDef RNA, and total RNAs were prepared for qRT-PCR analysis. Actin was used as an internal control. Each bar represents the mean ± S.E. (n=3). Means with an asterisk are statistically different ($P<0.05$) (Duncan's Multiple Range Test) among treatments; Means with two asterisks are statistically different ($P<0.001$) (Duncan's Multiple Range Test) among treatments. DEPC: diethyl pyrocarbonate.

the positively charged AMPs and the negatively charged microbial membrane components, which include LPS in Gram-negative bacteria and peptidoglycan in Gram-positive bacteria. But interaction mechanism of anionic defensins is not well understood and future research is needed to elucidate the mechanism.

Insect AMPs have been reported to be expressed predominantly in the fat body, the functional equivalent of the vertebrate liver, and increasing evidence shows that other tissues may also be involved in immune responses (Yi *et al.*, 2014; Gambichler & Skrygan, 2015). In the present study, after challenge with *B. thuringiensis*, expression of Px*Def* was significantly increased not only in the fat body, but also in the hemocytes, epidermis, and midgut. These results suggest that Px*Def* is systematically induced and may play its role in the defense against *B. thuringiensis* infection. *B. thuringiensis* is one of the popular pesticides for biological control of Lepidopteran pests including *P. xylostella*, which has developed a high resistance to *B. thuringiensis*. In the present paper, the expression of Px*Def* was analyzed after *B. thuringiensis* challenge using qRT-PCR method. The transcription levels of Px*Def* were lower after live *B. thuringiensis* infection than injection of heat-inactivated *B. thuringiensis*. In addition RNAi silencing of Px*Def* caused increase in mortality of *P. xylostella* larvae after *B. thuringiensis* infection. These results suggest that defensin may play an important role in *P. xylostella* against invading *B. thuringiensis*.

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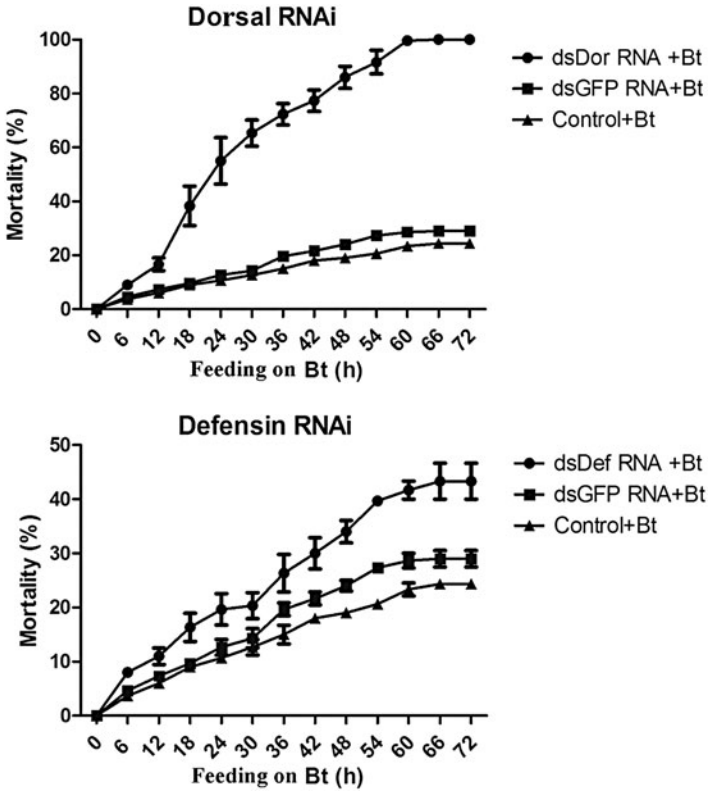


Fig. 4. Effect of knockdown expression of Px*Def* and Px*Dor* on susceptibility of *P. xylostella* larvae to *B. thuringiensis* (Bt). After dsRNA treatments, the larvae were treated with 200 ppm of Bt. Mortality was recorded every 6 h.

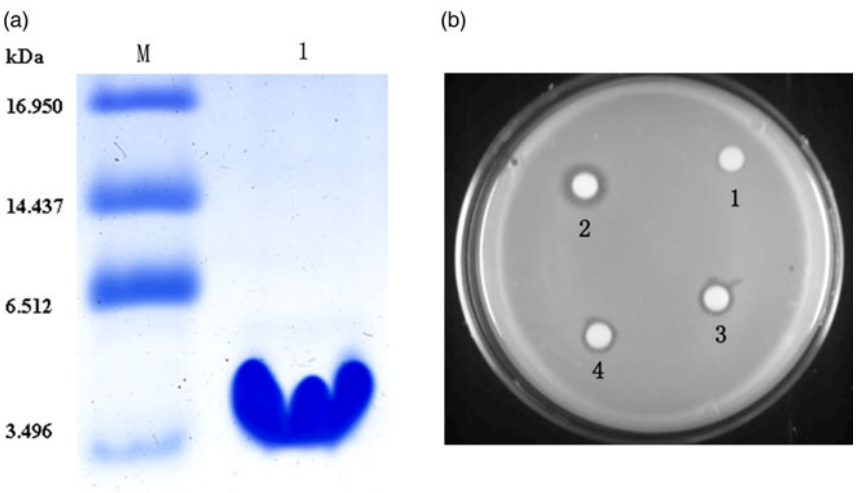


Fig. 5. Activity of recombinant *Px*Def against *B. thuringiensis*. (a) Tricine-SDS-PAGE analysis of purified recombinant *Px*Def. Lane M: polypeptide SDS-PAGE molecular weight standards; lane 1: 2 µg of purified recombinant PxDef. (b) Activity of recombinant PxDef against *B. thuringiensis*. Recombinant PxDef was tested for antibacterial activity against *B. thuringiensis* using a radial diffusion assay. The inhibition zone was quantified by measuring distance from the edge of the paper disk to the edge of the clear zone. Each experiment was repeated three times. 1, the inhibition zone of 10 µl PBS; 2, 10 µl (0.5 µg) Ampicillin, the inhibition zone is approximately 0.7 cm; 3 and 4, 10 µl (0.5 µg) recombinant PxDef, the inhibition zone is about 0.5 cm.

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Table 2. The activity of recombinant PxDef against microorganisms.

Microorganisms	PxDef MIC (μM)
Gram-positive	
Staphylococcus aureus	4.1
Bacillus cereus	3.6
Bacillus thuringiensis	1.6
Bacillus subtilis	2.6
Bacillus pumilus	3.1
Gram-negative	
Escherichia coli DH5α	35
Pseudomonas fluorescent	30
Salmonella choleraesuis	31
Fungi	
Metarhizium anisopliae	4.5
Botrytis cinerea	15.0
Penicillium crustosum	13.0
Peronophythora litchi	14.0
Colletotrichum gloeosporioiees Penz.	17.3
Colletotrichum orbicucar	12.5
Fusarium oxysporum	8.0

MIC, minimum inhibition concentration.

Insect immune related genes are regulated by the Toll and Imd pathways after microbial infections (Hoffmann & Reichhart, 2002). RNAi results showed that the expression of Px*Def* was significantly reduced after RNAi silencing of Dorsal as compared with the control (DEPC H_2O and GFP-dsRNA). This result suggests that the expression of Px*Def* is regulated by the Toll-Dorsal signal pathway. Whether expression of Px*Def* is also regulated by the Imd pathway or not remains to be determined.

RNAi silence of Dorsal in *P. xylostella* also down regulates the expression of Cecropins (Ouyang *et al.*, 2015) and lysozymes and may be many other immune-related genes regulated by the Toll-Dorsal pathway. This may account for almost 100% mortality in *P. xylostella* larvae treated with dsRNA to Dorsal and after feeding on live *B. thuringiensis*, since many immune effectors are down regulated after RNAi silencing of Dorsal, resulting in high susceptibility of larvae to live *P. xylostella*.

Supplementary material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0007485316000596.

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Disclosure

The authors of this study declare that there is no conflict of interests in this work.

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