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Short communication

Tribolium castaneum immune defense genes are differentially expressed in response to *Bacillus thuringiensis* toxins sharing common receptor molecules and exhibiting disparate toxicity

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ABSTRACT

In *Tribolium castaneum* larvae we have demonstrated by RNA interference knockdown that the *Bacillus thuringiensis* Cry3Ba toxin receptors Cadherin-like and Sodium solute symporter proteins are also functional receptors of the less active Cry3Aa toxin. Differences in susceptibility to *B. thuringiensis* infection might not only rely on toxin–receptor interaction but also on host defense mechanisms. We compared the expression of the immune related genes encoding Apolipophorin-III and two antimicrobial peptides, Defensin3 and Defensin2 after *B. thuringiensis* challenge. All three genes were up-regulated following Cry3Ba spore–crystal intoxication whereas only Defensins gene expression was induced upon Cry3Aa spore–crystal treatment, evidencing a possible association between host immune response and larval susceptibility to *B. thuringiensis*. We assessed the antimicrobial activity spectra of *T. castaneum* defensins peptide fragments and found that a peptide fragment of Defensin3 was effective against the human microbial pathogens, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, being *S. aureus* the most susceptible one.

1. Introduction

The entomopathogenic bacterium *Bacillus thuringiensis* (Bt) is the most widely used commercially successful biological control agent. The mechanism of pathogenesis of Bt against insects is a complex process in which toxin interaction with larval midgut receptors is a key step since resistance to Bt toxins is mainly due to mutations in the toxin binding region of the receptors or changes in the amount of binding sites (Pardo-López et al., 2013). The best characterized Bt toxin receptors are cadherin-like proteins (Fabrick et al., 2009; Vadlamudi et al., 1995) and aminopeptidase-N proteins (Knight et al., 1994), although other candidates, such as alkaline phosphatase and glycolipids, have been reported in several insect orders and nematodes (Griffitts et al., 2005; Jurat-Fuentes and Adang, 2004). Moreover, activation of the insect protective immune response to pathogens has been regarded as another contributing tolerance factor against Bt (Rahman et al., 2004).

Insect immunity comprises a suit of constitutive responses that rely on insect hemocytes and several rapid activated enzyme cascades such as phenoloxidase (PO), as well as inducible components such as antimicrobial peptides (Park et al., 2010; Tsakas and Marmaras, 2010). Coleopteran insects have recently become an invaluable source to the study of the innate immunity as they have a robust immune system and have retained many ancestral vertebrate genes that are not present in other invertebrates (Ntwasa et al., 2012). *Tribolium castaneum* (Tc), a major global pest of stored grain for human consumption (Phillips and Throne, 2010), is susceptible to Bt toxins and is a coleopteran model whose complete genome has been sequenced. Therefore, Tc constitutes an appropriate subject to study the innate immune response in relation to Bt toxins mode of action.

Bt Cry3Ba spore–crystal mixtures are active against Tc larvae (Contreras et al., 2013a) and a Cadherin-like and a Sodium solute symporter proteins have been reported as Cry3Ba functional receptors in this insect (Contreras et al., 2013c). In response to intoxication with Cry3Ba spore–crystal mixtures Apolipophorin-III (ApoLp-III) gene expression was induced and this protein was shown to be involved in regulation of PO enzyme activity in Tc (Contreras et al., 2013b).

Tamez-Guerra et al. (2008) reported that exposure to Bt toxins also increased expression of antimicrobial peptide (AMP) genes in *Trichoplusia ni* and they hypothesized that it might result in differences in susceptibility to the infection. AMPs are released upon pathogenic insult, and, in infected insects, accumulate in the hemolymph and kill pathogens directly by disrupting their membranes using mechanisms that are not fully understood but are linked

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to their ability to interact with the phospholipid bilayer due to an overall positive charge, hydrophobicity and amphipathicity (Brandenburg et al., 2012). AMPs are effective against a wide range of pathogens and they show a different mode of action than other antimicrobials, such as antibiotics. Intensive use of antibiotics has contributed to the selection of resistant bacterial strains that constitute a serious threat to human health. To overcome this problem, among other antimicrobial candidates, AMPs gradually emerge as important targets for the development of therapeutic products. An inherent part of the mechanism of AMP antimicrobial activity is the non-specific interaction with microbial cell membranes together with the short time-frame of interaction which decrease the probability of resistance development (Fernebro, 2011).

The aim of this work was to analyze the bases of differences in susceptibility to Bt spore–crystal mixtures exhibiting differential activity against Tc larvae (mixture containing Cry3Ba toxin more active than that containing Cry3Aa toxin). On the one hand we assessed whether Cry3Ba and Cry3Aa share the same receptor molecules and on the other hand, we compared the expression of defense related genes encoding Apolipophorin-III and two AMPs, Defensin3 and Defensin2 following intoxication with one of each spore–crystal mixtures. Finally, we analyzed the antimicrobial spectra of two *T. castaneum* Defensin3 and Defensin2 peptide fragments, evaluating their activity against the three human microbial pathogens, *Escherichia coli*, *Staphylococus aureus* and *Candida albicans*.

2. Materials and methods

2.1. Insects

A laboratory colony of Tc Ga-2 line insects were reared on whole-grain flour with 5% brewer yeast powder at 30 ± 1 °C in the dark.

2.2. B. thuringiensis *spore–crystal mixtures production*

BTS1, BTS00125L and HD73 Bt Cry3Aa, Cry3Ba and Cry1Ac-producing strains were grown in solid sporulation medium (Stewart et al., 1981) at 30 ± 1 °C until complete autolysis and spore–crystal mixtures were prepared as described before (Contreras et al., 2013a).

2.3. Human microbe strains and media

The antimicrobial activity of Tc defensin peptide fragments was tested in the following strains: *Escherichia coli* (CECT101), *Staphylococcus aureus* (CECT4013) and *Candida albicans* (CPA-2). *E. coli* and *S. aureus* were grown in LB medium (peptone 1%, yeast extract 0.5% and NaCl 1%) and *C. albicans* was grown in YPD medium (yeast extract 1%, peptone 2% and glucose/dextrose 1%) at 37 °C.

2.4. RNAi

The entire protocol for RNAi experiments was carried out as in Contreras et al. (2013c). RNA was isolated from 10- to 14-day-old larvae (after egg laying) and cDNA synthesis and PCR amplification using specific primers generated from TcCad1, TcSSS and TcAPN-I were performed. dsRNA was obtained by *in vitro* transcription and was administered to Tc larvae by ventral injection. Control larvae were injected with injection buffer (1.4 mM NaCl, 0.07 mM Na_2HPO_4, 0.03 mM KH_2PO_4, 4 mM KCl). Following injection, larvae were grown under standard rearing conditions.

TcCad1, TcSSS and TcAPN-I transcript levels were evaluated 8 days after dsRNA injection by semi-quantitative PCR using specific primers and TcSYN6 (*syntaxin 6*, XM_962400) gene expression was included as an endogenous control (see oligonucleotide sequences of primers in Table S1). Non silenced larvae were analyzed for comparison.

2.5. Toxicity assays

Toxicity assays on Tc larvae were performed using preweighed 10- to 14-day-old larvae (after egg laying), fed for 7 days on 20 µL flour disks (20% flour, w/v), prepared as described by Xie et al. (1996), containing 12.5 µg of Cry3Aa, Cry3Ba or Cry1Ac spore–crystal mixture per microliter flour disk for treatments or water in control assays. The assays were performed in 96-well polystyrene plates (Sterilin, Thermofisher) with one flour disk and one larva per well. Thirty larvae were used in each assay, and at least two replicates were carried out. Mortality was recorded after 7 days under laboratory rearing conditions.

For toxicity assays in gene silencing experiments, 4 days after dsRNA or control buffer injection, larvae were weighed and exposed to 12.5 µg/µL Cry3Aa spore–toxin mixture in flour disks. Flour disks prepared with water were used as controls of the toxicity assays.

2.6. Quantitative real-time PCR (qRT-PCR)

For immune-related gene expression analysis of ApoLp-III and Defensins 3 and 2, qRT-PCR was performed using cDNA (50 ng and 200 ng, respectively) synthetized from RNA isolated from 10- to 14-day-old larvae (after egg laying), in the conditions described in Contreras et al. (2013b), using specific primers (Table S1) and SYBR® Green detection (Life Technologies). For each sample, at least three biological replicates were analyzed using the mean values of three technical replicates. Gene expression was normalized using RPS18 (ribosomal protein S18, XM_968539) expression as an endogenous control (primers included in Supplementary Table S1) and gene expression relative-fold was calculated with the comparative Ct (ΔΔCt) method, using the StepOne software (Applied Biosystems). The data were analyzed by Student's *t* test for statistically significant differences ($p < 0.05$).

2.7. Synthetic peptides

Two 29 amino acid synthetic defensin peptides corresponding to annotated Tc Defensin 3 and 2, named as TcDef3-pep and TcDef2-pep peptides, respectively were obtained from GenScript. Peptide purities determined by analytical reversed phase C18 HPLC (Alltima™ C18, 4.6 × 250 mm; Grace & Co) were higher than 90% based on the peak areas detected by absorbance at 220 nm (Fig. S1). Peptide masses were confirmed by LC–ESI–MS (Fig. S2).

2.8. Analysis of the antimicrobial activity by FACS

Overnight bacteria and yeast cultures were diluted in phosphate-buffered saline (PBS) (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl), pH 7.4, to a final concentration of 1×10^6 cfu/100 µL. Cultures were mixed with 5, 10, 15, 20 and 25 µg/mL of two 29 amino acid Tc synthetic defensin peptides and incubated with shaking for 8 h at 37 °C. The β-3 human defensin (PeptaNova) was also assayed as a positive control. Cells were labeled with a 1:25 dilution of SYBR® green (Life Technologies) incubating for 1 h at room temperature followed by addition of a 1:10 dilution of propidium iodide (Sigma) and incubation for 10 min at room temperature. Labeled samples were analyzed in a FACSVerse flow cytometer (Becton Dickinson) equipped with three lasers (blue 488 nm, red 640 nm, and violet 405 nm). Cells were recorded as dead when they were propidium iodide positive or alive when SYBR green positive. Non treated cells grown at the same concentration were used as controls. A minimum of 10,000 cells within the gated region were analyzed. All data analyses were performed using FlowJo software (ver 8.8.7, Treestar Inc.).