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Is decreased generalized immunity a cost of *Bt* resistance in cabbage loopers *Trichoplusia ni*?

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ABSTRACT

We studied the immune response to *Bacillus thuringiensis kurstaki* (*Btk*) in susceptible (*Bt*-RS) and resistant (*Bt*-R) *Trichoplusia ni* after exposure to low doses of *Btk* and injection with *Escherichia coli*. We measured the levels of resistance, the expression profiles of hemolymph proteins, the phenoloxidase (PO) activity, and the differential number of circulating hemocytes in resistant and susceptible individuals. Individuals from the *Bt*-RS line became more resistant following a previous exposure to sub lethal concentrations of *Btk*, but the resistance to *Btk* of the *Bt*-R line did not change significantly. Similarly the *Bt*-R strain showed no significant changes in any of the potential immune responses, hemolymph protein levels or PO activity. The number of circulating hemocytes was significantly lower in the *Bt*-R strain than in the *Bt*-RS strain. Exposure to *Btk* decreased the hemocyte counts and reduced PO activity of *Bt*-RS larvae. Hemolymph protein concentrations also declined significantly in the susceptible larvae continually exposed to *Btk*. Seven peptides with antibacterial activity were identified in the hemolymph of *Bt*-RS larvae after exposure to *Btk* and five were found in the *Bt*-R larvae. When exposed to a low level *Bt* challenge the susceptible strain increases in tolerance and there are concomitant reductions in hemolymph protein concentrations, PO activity and the number of circulating hemocytes.

1. Introduction

Bacillus thuringiensis (*Bt*), is a microbial insecticide widely used to control lepidopteran pests of crops and forests. Resistance has developed in several Lepidopteran species after extensive use in post-harvest grain storage facilities (McGaughey, 1985), in the field (Kirsch and Schmutterer, 1988; Tabashnik et al., 1990), in the greenhouse (Janmaat and Myers, 2003) and following exposure in the laboratory (Ferré and Van Rie, 2002). Without continued exposure to *Bt*, resistance is lost in many of these species. In the cabbage looper, *Trichoplusia ni*, most populations revert from resistant to susceptible status within several generations when not exposed to *Bt*, and this suggests that maintaining *Bt* resistance is transient, and potentially costly (Janmaat and Myers, 2003).

Commercially, *Bt*-toxins are delivered in two ways; through transgenic crops (Vaeck et al., 1987), or through the spraying of Cry-toxin formulations that often contain viable *B. thuringiensis* spores. Toxicity occurs after *Bt*-endotoxins are ingested, solubilized in the alkaline midgut, and then proteolytically cleaved to release the active endotoxin (Haider et al., 1986; Jaquet et al., 1987; Broderick et al., 2006). Once cleaved, the endotoxin binds to receptors on the midgut brush-border membrane vesicles (BBMV) (Hoffman et al., 1988; Zhang et al., 2005; Wang et al., 2007), which results in pore formation (Rausell et al., 2004), midgut paralysis (Gill et al., 1992; Pigott and Ellar, 2007), and cell death (Zhang et al., 2008). This midgut damage is thought to create a point of entry for enteric bacteria or *B. thuringiensis* to invade the hemocoel (Broderick et al., 2006). However, if the insect can interrupt or defer this complex mode of action at any step, either through behavioral (deterrence) or physiological mechanisms, *Bt* efficacy is likely to be reduced.

Resistance to *Bt*-endotoxins is due largely to mutations on midgut receptors that can be both unique to the insect, and specific to the endotoxins encountered (Van Rie et al., 1990; Pigott and Ellar, 2007). Because of this specificity, it is possible that the physiological costs of *Bt* resistance also will vary among host species. In greenhouse populations of *T. ni*, resistance appears to be due largely to a physiological incompatibility between the midgut BBMV receptors and the toxins they bind (Wang et al., 2007). These losses in binding are likely due to mutations in the toxin binding region of the receptors, but also may be due to changes in the abundance of receptors on the midgut epithelium. Other mechanisms, or combinations of mechanisms also may reduce the toxicity of *Bt* to resistant hosts. These alternate tolerance mechanisms include coagulation reactions that prevent solubilization of the *Bt*-toxins

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(Ma et al., 2005a), changes in the proteolytic processing of the toxins, and changes in pH of midgut lumen (Ma et al., 2005b). In addition, changes in immune system function have also been associated with *Btk* exposure (Tamez-Guerra et al., 2008; Rahman et al., 2004, 2007).

Immune responses by insects to microbial pathogens comprise humoral and cellular factors. The humeral component includes phenoloxidase mediated melanization, the production of proteases and bacteriolytic enzymes, as well as the synthesis of potent antimicrobial peptides (AMPs) (Boman, 1991; Brey et al., 1993; Gillespie and Kanost, 1997; Lowenberger, 2001). Phenoloxidase, an enzyme required for several aspects of insect development also catalyzes key steps in biochemical pathways that lead to the melanization of pathogens (Cotter and Wilson, 2002). Studies with the pyralid *Ephestia kueniella* have associated exposure to *Bt* with increases in phenoloxidase activity and induced tolerance to *Bt*-endotoxins (Rahman et al., 2004, 2007). Phenoloxidase, however, represents only one aspect of a potent and effective immune system (Soderhall and Cerenius, 1998; Gillespie and Kanost, 1997; Cerenius and Soderhall, 2004).

The cellular (hemocyte) response primarily involves the phagocytosis, nodulation or encapsulation of foreign microbes and debris (Lavine and Strand, 2002), but also may be involved in the rapid synthesis, expression and delivery of potent AMPs (Lavine et al., 2005). Hemocyte-mediated responses have been associated with significant changes in the number of circulating cells (Bidochka and Khachatourians, 1987; Nakahara et al., 2003), changes in abundance of particular hemocyte types, changes in phagocytic rates (Dubovskiy et al., 2008), and the differentiation of circulating cells (Strand, 2008). These different components of the insect's humoral and cellular immune system work in concert to eliminate microbial pathogens (da Silva et al., 2000).

Before a response can occur, however, the pathogen must be recognized by the insect immune system as non-self. In Gram-positive bacteria such as *B. thuringiensis*, peptidoglycan, a major constituent of the cell wall, is recognized by peptidoglycan receptors (PGRP) and this initiates a strong immune response (Leulier et al., 2003; Chang and Deisenhofer, 2007). These receptors are membrane bound or secreted into the hemolymph, such that if the midgut is breeched, or bacterial invasion of the hemocoel occurs, a rapid immune response takes place. What is unclear, however, is whether *B. thuringiensis* spores are recognized after ingestion, and if this can subsequently cause a systemic immune response that is detectable in the hemocoel.

In this study, we examined how *Bt*-resistant and -susceptible strains of *T. ni* differ in their immune response following exposure to low doses of a commercial formulation of *Btk* containing spores and five endotoxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry2Ab). In addition to the susceptibility of pre-exposed hosts to *Btk*, we measured several parameters of the insect immune system including hemolymph phenoloxidase activity, the number of circulating hemocytes, and the presence of antimicrobial peptides in the hemolymph. Given the findings of Wang et al. (2007), that the loss of binding of *Bt*-endotoxins to the midgut receptors could only account for high-levels of tolerance to Cry1Ab and Cry 1Ac, we predicted that components of the innate immune response might contribute to the overall, total resistance to spore-crystal formulations.

2. Materials and methods

2.1. Rearing of Bt-resistant and Bt-susceptible T. ni line

A *Bt*-resistant (*Bt*-R) *T. ni* colony was initiated from 90 individuals collected from a commercial tomato greenhouse in British Columbia, Canada in 2001 (Janmaat and Myers, 2003). The *T. ni* population at collection was found to be 113-fold more resistant in the first generation of laboratory culture than a reference susceptible laboratory colony. Two lines were established on a wheat-germ based diet (Ignoffo, 1963) and reared at 26 °C, a 16:8 (L:D) photoperiod. One line was reared without any *Btk* exposure and exhibited a significant decrease in resistance (change in LC_{50} from 256 to 2.7 kIU ml^{-1} diet where kIU is equal to 1000 International Units of *Bt* activity) (Janmaat and Myers, 2003). This is referred to as the reverted-susceptible line (*Bt*-RS). The resistant (*Bt*-R) line was exposed to *Btk* (DiPel WP, Valent Biosciences) periodically during laboratory culture to maintain resistance as described (Janmaat et al., 2004).

2.2. Bt-induction bioassay: changes in susceptibility

The induction bioassay was repeated three times within the period of a year. In the first and third replicates, a response to pre-exposure was evaluated in both *Bt*-R and *Bt*-RS strains. In the second replicate, a pre-exposure response was evaluated only in the *Bt*-RS line. In all experiments, groups of 25 *T. ni* larvae were reared in 175 ml Styrofoam cups containing 15–20 ml of artificial diet. After 4 days of growth, larvae in the treatment group were transferred to cups containing 10 ml of a *Btk*-diet mixture with a sublethal *Btk* dose (0.6 kIU ml^{-1}) diet as defined by preliminary experiments. Larvae in the control group were transferred to cups containing 10 ml of artificial diet without *Btk*, in the first two replicates of the induction assay. In the third replicate, larvae in the control group were not transferred but were maintained on *Bt*-free diet. Following 18–24 h on the pre-treatment dose of *Btk*, larvae were transferred in groups of five to 59.2 ml plastic soufflé cups (Solo Cup Company, Highland Park, IL, USA) containing 3 ml of fresh diet with a range of *Btk* concentrations (0.625–200 kIU ml^{-1}) or a control containing no *Btk*. Larval mortality was observed 3 days following exposure to treatments and susceptibility to *Btk* was determined by probit analysis of a concentration-mortality assay as described previously (Janmaat and Myers, 2003). Unique dose ranges were required for each line due to their inherent differences in susceptibility to *Btk*.

2.3. Phenoloxidase assay and hemolymph protein concentration

Additional *T. ni* larvae were raised as described above, and after 4 days of growth were transferred in groups of 20 larvae to 175 ml Styrofoam cups with the appropriate diet treatment. Control larvae were transferred to freshly prepared artificial diet without *Btk*, and larvae in the *Bt*-exposure group were transferred to fresh diet containing *Btk*. We collected hemolymph from these insects in the fifth larval instar. Because the treatment groups developed at different rates, hemolymph samples were collected at different times. Hemolymph samples were collected by first excising a proleg with fine scissors and allowing the exuding hemolymph to pool onto parafilm. Ten-µl of this hemolymph were diluted in 240 µl of Dulbecco's phosphate buffer saline (DPBS) and samples were frozen at −20 °C for 24–48 h to disrupt hemocyte membranes (Wilson et al., 2001). Triplicate 50 µl hemolymph/buffer mixtures were transferred into 96-well microtitre plates and 150 µl of 15 mM dopamine HCl (Sigma-Aldrich, St. Louis, MO, USA) were added to each well. Absorbance was measured at 492 nm on a Spectramax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Preliminary experiments indicated that the linear phase of the reaction began shortly after the addition of dopamine and continued for 40 min. The kinetic activity of phenoloxidase per µl hemolymph sample was expressed as change in optical density units per minute ($\delta OD\ min^{-1}$), at VMAX.

Hemolymph protein concentrations were measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) used as a protein standard. Trip-