Resistance against *Bacillus thuringiensis* endotoxins in a laboratory population of *Helicoverpa armigera* is based on an elevated immune status.

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ABSTRACT

Recent observations suggest that lectin-like toxins, such as endotoxins from *Bacillus thuringiensis* are sequestered inside the gut of immune-induced insects providing tolerance to Bt-formulations. To investigate the induction and sequestration process, we used toxin and lectins that bind to galactosamine (Gal) and N-acetylagalactosamine (GalNAc) to identify binding sites in the midgut of induced and non-induced insects using confocal microscopy. Our observations suggest that immune and metabolic hemolymph components are transported across the gut epithelium and accumulate inside the gut lumen in Bt-tolerant insects. Co-location of immune components and toxin indicates that some of the toxin is inactivated by coagulation reactions inside the gut lumen before it can reach the brush border membrane.

Key words: *Bacillus thuringiensis*, endotoxin, Bt-resistance, immune response, lectin

INTRODUCTION

Bacillus thuringiensis endotoxins (Bt-toxins) are the most important biopesticides used in controlling insect pests and vectors of disease. Bt-toxins are extensively used because of a relative target-specific mode of action, low risk to human health and environmentally benign properties. Thus the potential for resistance to Bt-toxin in insect species is a serious threat to human health and agricultural production. One of the unintended outcome of continuous intensive pest control measures have been the emergence of new tolerance mechanisms in pest insects. For example, the selection pressure imposed on insect populations in transgenic crops expressing *B. thuringiensis* toxin proteins requires continuous monitoring to prevent insects with target site mutations in toxin receptor genes from becoming homozygous. Resistance management strategies, such as the so-called refuge strategy (Bates *et al.* 2005), are based on the precondition that heterozygous mutant pest insects are killed by toxin levels expressed in transgenic plants. Although most target site mutations are recessive, heterozygote insects are frequently semi-dominant (or incomplete recessive), which has now become a major issue in resistance management. Two reasons are given for the observed semi-dominance: One is haplo-insufficiency, where the gene products from one chromosome are not sufficient to produce complete susceptible phenotypes. While the resistance level caused by haplo-insufficiency is related to the function of the mutant gene locus, it is independent of the metabolic status or selection pressure of the insect population. A second reason for incomplete recessive phenotypes appears to be separate from target site mutations in the form of dominant multi-gene traits providing protection to low to medium pesticide levels. These metabolic tolerance mechanisms are difficult to study and, given that they provide only a small proportion of the resistance level usually achieved by target site mutations, where mostly ignored until recently.

A possible mechanism for the sequestration of toxins is the aggregation of immune components around the emerging toxin inside the gut lumen. Endotoxins from *B. thuringiensis* have lectin-like properties (Knight *et al.* 2004), raising the possibility that the mature toxin interacts with glycosylated immune components that are secreted into the gut lumen of immune-induced insects. Since the amount of the pro-coagulant lipophorin is increased in tolerant larvae (Rahman *et al.* 2007) and more phenoloxidase is transported from the hemolymph into the gut in induced larvae (Sarjan *et al.* submitted), we wanted to investigate the transport and interaction of these components in induced and non-induced

larvae. If immune components are glycosylated in such a way to bind mature toxin, an aggregation reaction would occur with a oligomeric toxin complexes. Here we applied mature toxin and lectins with toxin-like sugar-specificity to identify possible binding sites in the gut lumen of induced insect larvae. Using confocal microscopy we show that mature Bt-toxin co-localises with immune components in Bt-tolerant larvae. This could indicate that the toxin emerging inside the gut lumen causes an aggregation reaction that potentially inactivates the toxin before it can reach the brush membrane of the gut lining.

METHODS

Insect strains

A laboratory strain (BX) of *Heliothis. armigera* was selected for resistance to Cry1Ac and subsequently back-crossed with a susceptible (ANGR) strain to provide a isogenic resistant (ISOC4) strain (Akhurst *et al.* 2002, Akhurst *et al.* 2003), which has recessive resistance and tolerance due to an elevated immune status (Ma, 2005). A susceptible laboratory population of diamondback moth (DBM), *Plutella xylostella* has been maintained on cabbage seedling leaves (*Brassica oleracea* var. *capitata* cv. DBM larvae were immune-induced by feeding leaves coated with a sublethal dose of Bt-formulations (50ppm).

Insects were maintained, and bioassays conducted, at 22ºC under a 14:10 light:dark photoperiod. Larvae were reared on an artificial diet as described {Ma 2005).

Bt-toxin

The Cry1Ac toxin used as a probe was purified from *B. thuringiensis* subsp. *kurstaki* HD73 by sucrose gradients. Purified crystals were suspended in a solution containing 30 mM Na_2CO_3 and 1% mercaptoethanol at pH 9.6 and digested with gut juice extracts from *Pieris rapae*. Mature toxin was obtained by incubating protoxin and gut juice (100:1 protein ratio) at room temperature and monitoring aliquots every few hours using electrophoresis.

Antibodies

Antisera against the 230 kDa (putative apolipoprotein I) protein were obtained by immunizing rabbits against the SDS-PAGE-purified protein. Gel pieces containing the protein were cut from Coomassie-stained poly-acrylamide gel and crushed in the presence of Freund's adjuvant. Immunisation was performed according to standard procedures with one injection using complete Freund's adjuvant followed by three booster injections at four-week intervals with an incomplete Freund's adjuvant. About 10µg of the gel-purified proteins were used for each injection. The antiserum was recovered two weeks after the fourth injection. Antibodies against Cry1Ac were obtained in rabbits by the same procedure and affinity purified. Serum was used in 1:1000 dilutions and purified antibodies used in 1:100 dilution. Antibodies against prophenoloxidase (PPO) were kindly provided by M. Kanost (Jiang *et al.* 1997).

Insect gut staining

Gut tissues were dissected from caterpillars and midgut sections were separated with microscissors leaving peritrophic membranes intact. The tissues were fixed in paraformaldehyde (4%), containing 0.5% Tween 20 in the presence or absence of phenylthiourea (PTU) for several hours. After washing overnight, tissues were incubated for at least four hours with gut juice-activated Cry1Ac (ca. 10µg/ml), antibodies against Cry1Ac (1:1000 dilution), and fluorescein (FITC)- or tetramethyl rhodamine (TRITC)-conjugated secondary antibodies (1:5000 dilution), followed by four washing steps each. Lectins (conjugated with FITC or TRITC) were applied (0.1µg/ml) together with the secondary antibodies in double-staining experiments using different combinations of fluorescent lectins and antibodies. Peanut agglutinin (PNA), which co-localised with Cry1Ac, PPO- and lipophorin-antibodies to granule-like structures, was used as a reference probe to determine the localization of FITC-conjugated secondary antibodies in various antibody treatments. To keep non-specific staining to a minimum, fixation and incubations were performed in the presence of PTU. Focal planes during confocal microscopy were chosen to be perpendicular to the longitudinal tube of the dissected midgut and sufficiently interior to be separated from the autofluorescense of the peritrophic membranes (Figure 1).

RESULTS AND DISCUSSION

Transport across the gut lining

Since mature endotoxins from *B. thuringiensis* bind to Gal- and GalNAc-containing glycoproteins (Knight *et al.* 2004) and glycolipids (Griffitts *et al.* 2005), we examined the binding of corresponding lectins using confocal microscopy. While the GalNAc-specific HPL showed extensive staining in the hemolymph on the hemocyte surface and soluble plasma components, relative little staining was observed with the Gal-specific PNA, except in granules inside hemocytes (data not shown). In contrast, with the exception of the peritrophic membrane little HPL-staining was observed inside the midgut, whereas PNA showed high levels of staining in immune-induced larvae (see below).

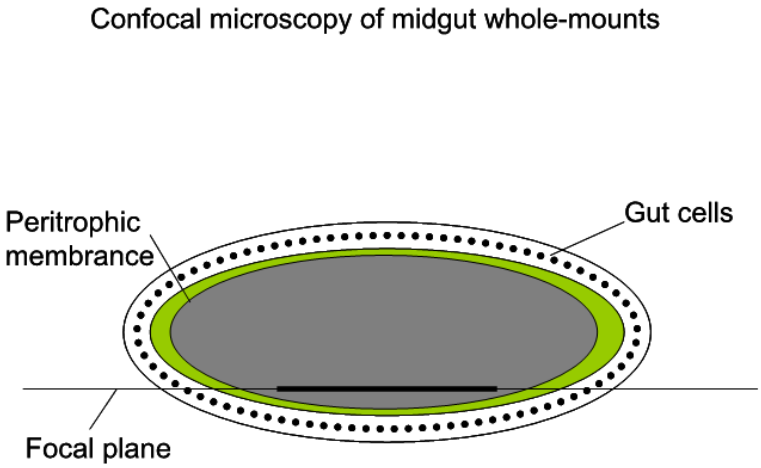

Figure 1. Schematic depiction of focal planes in confocal microscopic inspection of midgut whole-mounts. Focal planes for assessment of gut contents were perpendicular to the longitudinal tube of the dissected midgut and sufficiently interior to be separated from the autofluorescense of the peritrophic membranes.

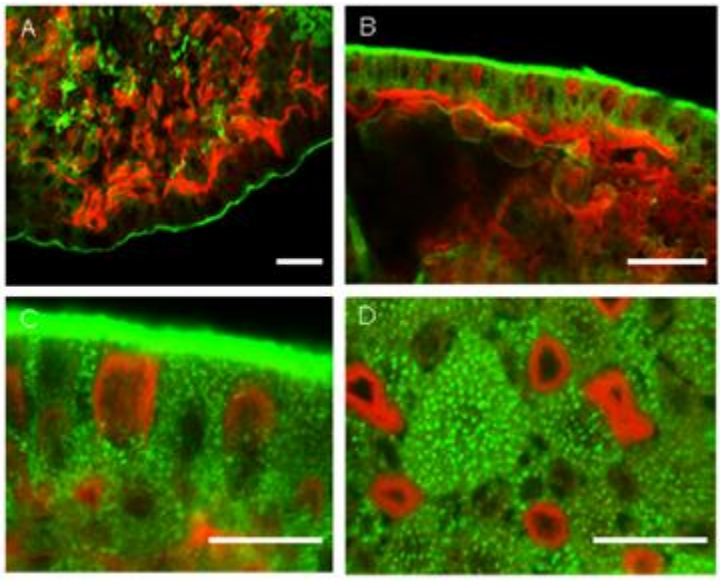

Figure 2. Midgut sections from induced DBM larvae stained with FITC-conjugated HPL. Different magnifications of optical sections cutting perpendicular and alongside epithelial gut cells (A-C) and across epithelial cells (D). C) and D) are the same magnification. A) HPL stains granular and smooth structures inside the gut lumen mainly at the peritrophic membrane. The lack of staining in cells is an optical shielding effect of the strong labelling of the basement membrane lining the hemocoel. B) At higher magnification HPL staining of cells becomes visible below the strong basement membrane staining. C). and D) HPL stains vesicular structures mostly inside and less at the interface of gut cells. The peritrophic membrane is visible in TRITC-eliciting wavelength as red autofluorescence. Note the smooth (non-granular) HPL staining around the peritrophic membrane, and in the epithelial cells towards the peritrophic membrane.

To investigate this puzzling observation further we examined the lectin-staining at the gut epithelium, where hemolymph plasma components, such as lipid particles and phenoloxidase are transported across into the gut lumen.

When GalNAc-specific HPL-binding to the gut cells was analysed, most of the staining was observed inside the gut cells in structures resembling transport vesicles or Golgi complexes (Figure 2). Apart from a faint staining of the peritrophic membrane little HPL-staining was observed in the gut lumen. Conversely, when Gal-specific PNA-binding was examined on similar sections, PNA-staining was located in the gut-proximal part of gut cells (Figure 3) and inside the gut lumen (see below). This could indicate that immune components are transported through gut cells by vesicular transport and modified on the transit by changing glycosylation from GalNAc- to Gal-binding properties.

Toxin interaction with immune components

To examine immune components inside the gut and to investigate a possible role in the aggregation of toxins, we performed confocal sections in a *Helicoverpa armigera* strain (ISOC4), which shows an elevated immune status in conjunction with resistance to the toxins (Ma *et al.* 2005). When the gut content of ISOC4 was compared with a susceptible strain (ANGR), we observed grey peritrophic membranes and dark melanized gut debris in more than fifty percent of ISOC4, but not in ANGR larvae (Ma *et al.* 2005). Higher melanization reactions were also observed in protein extracts from gut tissue and gut content in ISOC4 compared to ANGR larvae (data not shown).

Confocal microscopic analysis indicated that Cry1Ac-binding in the gut lumen (Figure 4) co-localized with anti-lipophorin antibodies (Figure 5) and anti-PPO antibodies (Figure 6) in small globular structures, which were more abundant in ISOC4 insects. Since lipophorin in hemolymph is a pro-coagulant (Li *et al.* 2002) and melanization is associated with phenoloxidase activity (Kanost *et al.* 2004), this indicates that Cry1Ac binds to components in the gut lumen involved in coagulation or melanization.

In summary, the tolerance to Bt-toxins in immune-induced insect larvae may be based on an elevated immune status. Immune-induction enhances the transport of immune components, such as lipophorin and phenoloxidase from the hemolymph into the gut lumen. These components are transported through the gut cells in vesicles and may undergo glycosylation modifications by passing through Golgi vesicles.

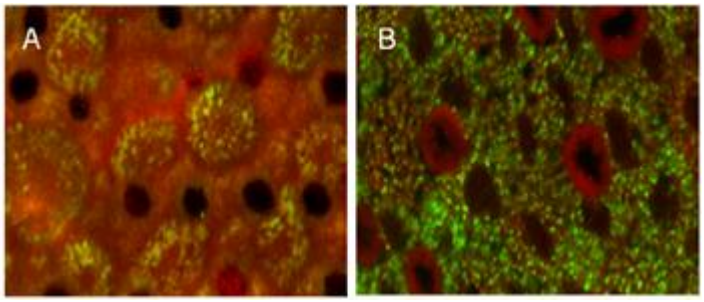

Figure 3. Midgut section stained with FITC-conjugated PNA (A) and FITC-conjugated HPL (B). Focal planes cut longitudinal gut cells in a perpendicular traverse axis showing Golgi and other vesicles.

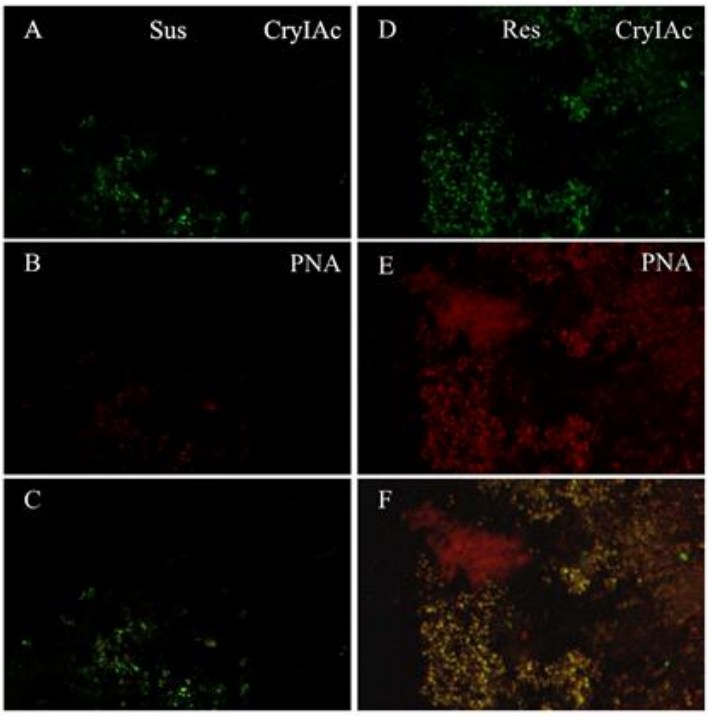

Figure 4. Binding of activated Cry1Ac in the gut lumen. Gut sections of 3rd instar *H. armigera* larvae were incubated with gut juice-activated Cry1Ac and bound toxin was identified with antibodies against Cry1Ac. TRITC-conjugated peanut agglutinin (PNA) was added together with the FITC-conjugated secondary antibody and inspected under indirect UV-light using confocal microscopy. In tissues treated with preserum or FITC-conjugated secondary antibodies alone, staining to dot-like structures was less than 10% (not shown). **A**) Cry1Ac-staining in gut lumen of susceptible (Sus) larvae. **B**) PNA-staining in gut lumen of susceptible (Sus) larvae. **C**) Overlay of Cry1Ac- and PNA-staining in gut lumen of susceptible (Sus) larvae. The Cry1Ac-stained dots outnumber PNA-stained dots, which could indicate that toxin molecules bind to other target sites than dot-like structures. **D**) Cry1Ac-staining in gut lumen of resistant (Res) larvae. **E**) PNA-staining in gut lumen of resistant (Res) larvae. **F**) Overlay of Cry1Ac- and PNA-staining in gut lumen of resistant (Res) larvae. In the gut lumen of resistant insects the toxin binds predominantly to dot-like structures. Note the orange colour of dot-like structures in the overlay indicate a co-location of staining.

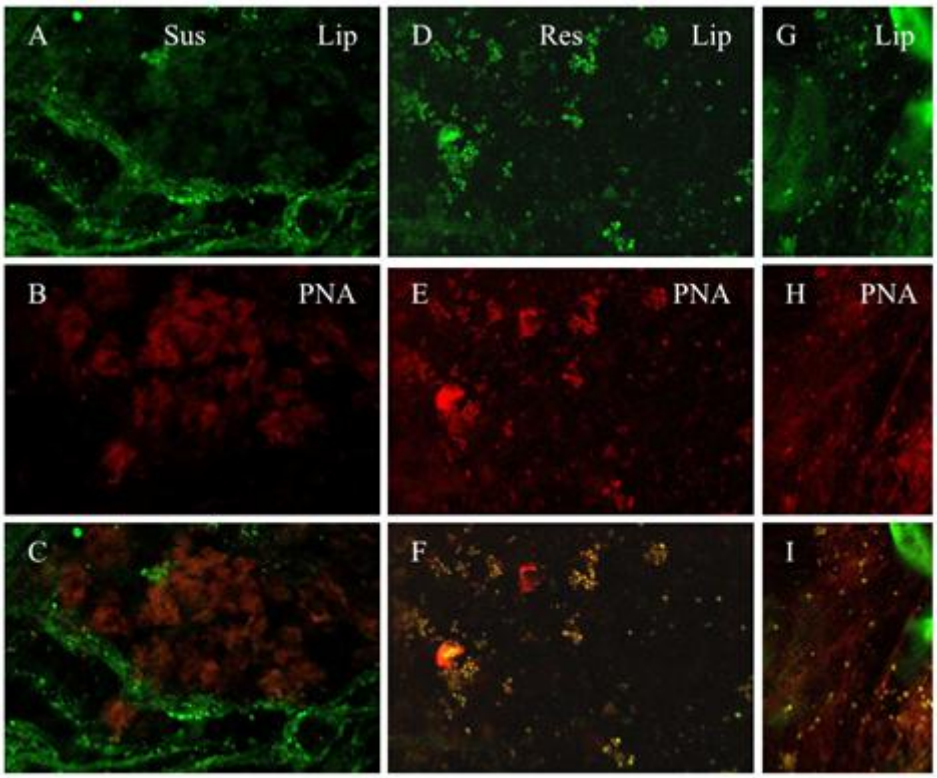

Figure 5. Presence of lipophorin in the gut lumen around the peritrophic membrane. Gut section of 3rd instar *H. armigera* larvae were incubated with antibodies against lipophorin, stained with FITC-conjugated secondary and inspected under indirect UV-light using confocal microscopy. TRITC-conjugated peanut agglutinin (PNA) was added together with the secondary antibodies. Dot-like structures were less than 10% in tissues treated with preserum or FITC-conjugated secondary antibodies alone (not shown). **A**) Lipophorin-staining in the gut lumen and on the peritrophic membrane of a susceptible (Sus) caterpillar **B**) PNA-staining of the same microscopic section **C**) Overlay of lipophorin and PNA-staining. **D**) Lipophorin-staining in the gut lumen and on the peritrophic membrane of a resistant (Res) caterpillar **E**) PNA-staining of the same microscopic section. **F**) Overlay of lipophorin and PNA-staining. **G**) Lipophorin-staining on a peritrophic membrane which was dissected from a resistant (Res) larvae. **H**) PNA-staining of the same microscopic section. **I**) Overlay of lipophorin and PNA-staining.

At this stage it is not known whether the enhanced coagulation, observed in immune-induced insects, is due to the sugar modifications or simply a result of increased amounts of pro-coagulant, which may increase the chances of aggregation reactions. Since melanization reactions are associated with reactive oxygen production (Nappi & Christensen 2005), it is conceivable that a toxin-binding pro-coagulant inactivates the toxin in the gut lumen before it can reach the brush border membrane.

These observations could indicate the presence in the gut lumen of immune molecules that may be more abundant and immune-reactive in the ISOC4 strain than in their susceptible counterparts, resulting in the inactivation or sequestration of mature toxin molecules in the gut lumen and thereby protecting the gut epithelium from the damaging effects of the toxin.

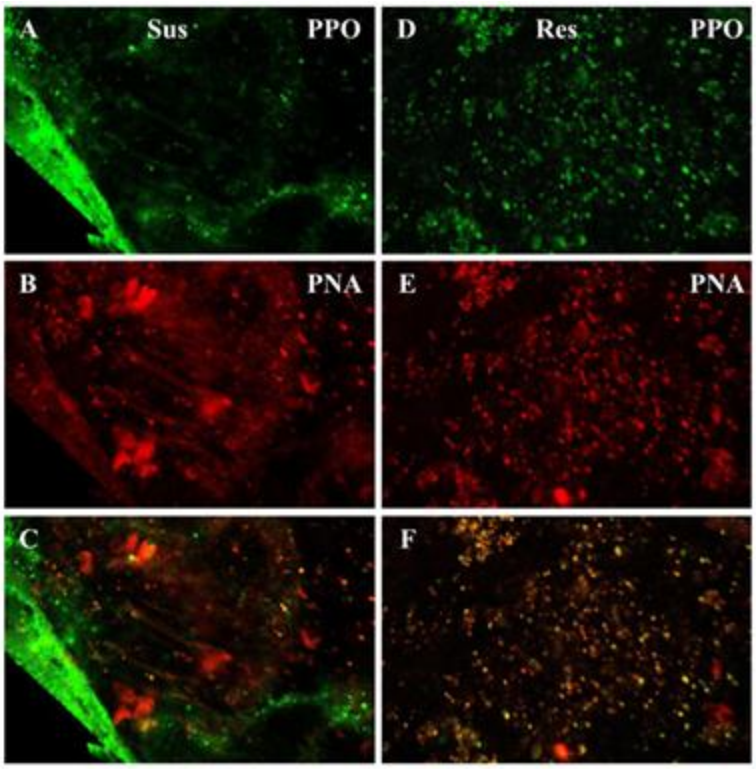

Figure 6. Presence of PPO in the gut lumen. Gut sections of 3rd instar *H. armigera* larvae were incubated with antibodies against PPO (Jiang et al., 1997), stained with FITC-conjugated secondary antibodies and inspected under indirect UV-light using confocal microscopy. TRITC-conjugated PNA was added together with the secondary antibodies. Staining of dot-like structures were less than 10% in tissues treated with preserum or FITC-conjugated secondary antibodies alone (not shown). **A**) PPO-staining in gut lumen of susceptible (Sus) larvae. **B**) PNA-staining in gut lumen of susceptible (Sus) larvae. **C**) Overlay of PPO- and PNA-staining in gut lumen of susceptible (Sus) larvae. **D**) PPO-staining in gut lumen of resistant (Res) larvae. **E**) PNA-staining in gut lumen of resistant (Res) caterpillar **F**) Overlay of PPO- and PNA-staining in gut lumen of resistant (Res) caterpillar.

The immune-reactive status of these Bt-resistant insects may resemble malaria-resistant mosquitoes, which have elevated melanotic activities compared to susceptible insects (Dimopoulos *et al.* 2000, Dimopoulos *et al.* 2002).

CONCLUSION

The tolerance to Bt-toxins in immune–induced insect larvae may be based on an elevated immune status. Immune-induction enhances the transport of immune componens, such as lipophorin and phenoloxidase from the hemolymph into the gut lumen. The observations suggest that immune and metabolic hemolymph components are transported across the gut epithelium and accumulate inside the gut lumen in Bt-tolerant insects. Co-location of immune components and toxin indicates that some of the toxin is inactivated by coagulation reactions inside the gut lumen before it can reach the brush border membrane.

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