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ARTICLE

Histopathology of the larval midgut of *Helicoverpa armigera* (Hübner) fed on *Bacillus thuringiensis* crystals and *Bt*-tomato plants

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Abstract The histopathological effects of the spore-crystal complex of indigenous *Bacillus thuringiensis* (*Bt*) isolate, as well as *Cry* 2Ab gene expressed in transgenic tomato plants on the midgut of 4th instar larva of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidea) has been investigated using the transmission electron microscope (TEM). Remarkable ultrastructural changes were observed in the columnar and goblet cells of the larval midgut after feeding on either transgenic tomato leaves, or spore-crystal complex of *Bt*. The effects observed included breakdown of microvilli of epithelial cells, increase in the electron density of the cytoplasm and vacuolation associated with different sizes of lysosomes; interruption of the goblet cells and distorted goblet cavities which lost their cytoplasmic projections; destruction of the mitochondria which lost their cristae; degeneration of the endoplasmic reticulum; collapse of the nucleus associated with rupture of nuclear envelope and clumped chromatin. Feeding the larvae on transgenic *Bt*-tomato plants caused in addition to the aforementioned changes severe vacuolation and degeneration of the nucleus in both columnar and goblet cells and the nuclear membrane was broken into electron dense ring spheres.

1. Introduction

Studies on the ultrastructural changes of lepidopterous larvae fed *Bt* endotoxin received the attention of many workers [9,19,20,5,23,13,21,8,14]. In their investigations, the interaction between *Bt* toxins and the midgut of lepidopterous larvae using purified crystal toxins, was evaluated. These studies showed that crystal endotoxin caused the destruction of both goblet and columnar cells of the mid-gut together with notable changes in the microvilli, mitochondria and membranes associated with the Golgi complex [4,7]. Therefore, a study has been done to investigate the effects of toxins expressed in transgenic cotton plants for comparison. In this concern, the

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histopathological effect of *Cry* 1AC toxin expressed by *Bt* – cotton plant fed to the lepidopterous insect *Alabama argillacea* (Hübner) was reported by Sousa et al. [22].

2. Materials and methods

2.1. Bacterial toxins

An indigenous *Bt* isolate containing *Cry* 2Ab; isolated from the soil of Qena governorate, Egypt was used [1]. Fourth instar larvae were isolated from the laboratory colonies of *Helicoverpa armigera* established on a semi-artificial diet [6]. The larvae were starved for several hours and then fed on a diet containing *Bt* at 500 µg/ml for 48 h. Similarly, another group of larvae of *H. armigera* were fed on transgenic *Bt* tomato plants for 1 day. These plants were developed via *Agrobacterium*-mediated transformation to express the insecticidal protein *Cry* 2Ab [18]. *Agrobacterium tumefaciens* LBA4404 strain harboring binary vector (*Cry* 2Ab gene) was used for tomato transformation. The plasmid contains insecticidal crystal gene (*Cry* 2Ab) under the transcriptional control of cauliflower mosaic virus (CaMV35S) promoter and nopaline synthase (NOS) terminator, and the selectable marker of hygromycin phosphotransferase gene (*hpt*) and *β*-glucuronidase (*gus*) as a reporter gene. Cotyledon and hypocotyl explants of 7–10 day-old seedlings were used. The transformation procedure was carried out as described by McCormick et al. [11], McCormick [10]. Putative transformed tomato plantlets were confirmed by histochemical analysis (GUS expression) and genomic analysis. Untreated larvae were used as control and reared on a semi-artificial diet. All experimental larvae were held at 28 ± 2 °C and 75% RH.

2.2. Electron microscopy

For electron microscopic investigations, the midgut of the control and treated larvae was dissected and fixed immediately in 2.5% glutaraldehyde sodium cocodylate buffer at pH 7.3 for 4–6 h, washed three times for 10 min each and kept in buffer for one day at 4 °C. Postfixation was done in 2% osmium tetraoxide buffer at pH 7.3 for 2 h at 4 °C (until the samples became dark in color), followed by 3 washes with glutaraldehyde buffer and kept for another day in this buffer at 4 °C. The tissues were subjected to dehydration in graded ethanol series (30–100%). Propylene oxide was substituted for the alcohol with two changes, of 2 min each. Embedding using 1:1 araldite–propylene oxide for 3 h, followed by the same mixture (2:1 ratio) for 6 h was carried out. Finally, the samples were transferred to a bath of fresh araldite and left overnight, and then the tissues were embedded in araldite-filled capsules. The capsules were allowed to polymerize at 4 °C for 2 h and transferred to an oven for 24 h at 45 °C and the temperature was then increased to 60 °C for another 24 h or until the blocks were adequately hardened [17,12]. Semi-thin sections 1–3 µ thick, were stained with alkaline toluidine blue for preliminary examination by light microscope and for proper orientation. Ultra sections prepared using (Reichert-Jung) microtome were mounted on copper grids of 100–150 mesh size and double-stained with uranyl acetate (30 min) and lead citrate (3–5 min) before examination. The sections were examined and photographed using TEM-ten Zeiss 60 kV, resolution about 5 A°.

3. Results

It appears that the wall of the midgut in the control *H. armigera* larvae consists of a single layer of epithelial cells including columnar and goblet cells lying on a basement membrane, which is attached to the muscle connective tissue. Each epithelial cell has numerous microvilli (MV) which appear as striated borders on the apical surface of the columnar cells and apical intermicrovillar crypts (AC) (Fig. 1A). The cytoplasm is packed with numerous organelles such as mitochondria (M) with clear matrix, membranes and cristae, small Golgi bodies (GB) with few saccules, free ribosomes (R), and rough endoplasmic reticulum (rER). A clear rounded nucleus (N), regular nuclear envelope (NE) and central nucleolus (Nu) with normal chromatin appear in Fig. 1B.

The goblet cells are calyx shaped, and they lie between the columnar cells (Fig. 1C). The goblet cell is characterized by a large cavity called goblet cavity (GC1, 2, 3), with an apical opening into the lumen of the midgut. This cavity is lined by numerous cytoplasmic projections extending inside; each containing a mitochondrion. The nucleus (N) of the goblet cell is flat in shape lying in the basal portion of the cell beneath the goblet cavity.

3.1. Histopathological changes in the midgut of larvae fed on a diet containing Bt endotoxin

The midgut of the larvae fed on *Bt* endotoxin showed degenerative appearance of columnar cells (1, 2, 3) associated with destroyed microvilli (MV) and a remnant of peritrophic membrane (Tm) (Fig. 2A). Also, all cytoplasmic organelles appear with numerous vacuoles (V). Mitochondria are clearly vacuolated, destroyed, and associated with different sizes of lysosomes (L). Moreover, shrinkage of the nucleus (N), destroyed nuclear sheath, vacuoles formation and clumped chromatin appear with accumulation of ribosomes.

The goblet cells suffered from degeneration in their cytoplasm (Fig. 2B). The microvilli (MV) were disrupted showing degenerative appearance at the apical region. The goblet cavity (GC1, GC2) appears losing its constituent organization. The cytoplasm is characterized by increased vacuolation (V) associated with different sizes of lysosomes (L) and destroyed mitochondria (M). An interrupted goblet cavity (GC) appears with cytoplasmic extensions scattered as mucigenous substances inside (Fig. 2C). Many vacuolated organelles on the periphery of the goblet cavity were observed. The nucleus, nuclear envelope, and nuclei lost their organization and clumped chromatin appeared inside (Fig. 2D).

3.2. Histopathological changes in the midgut of H. armigera larvae fed on transgenic Bt-tomato plants

When the larvae were fed on *Cry*2Ab, ultrastructural changes in the columnar and goblet cells occurred. Different significant changes were recorded in the microvillus, mitochondria, cytoplasmic vacuolation, and other parts (Fig. 2E). A sloughed part of degenerated epithelial cell appears (Slo) toward the