Chitinolytic enzymes from *Streptomyces albidoflavus* expressed in tomato plants: effects on *Trichoplusia ni*

Carmenza E. Gongora[1]*, Shaojiu Wang[1], Raymond V. Barbehenn[2] & Roxanne M. Broadway[1]

[1]*Entomology Department, NY State Agricultural Experiment Station, Cornell University, Geneva, NY14456, USA;*
[2]*Department of Biology, University of Michigan, Ann Arbor, MI 48109, USA; *Present address: CENICAFE, Plan Alto, Chinchina, Caldas, Colombia (E-mail: Carmenza.Gongora@cafedecolombia.com)*

Abstract

Tomato (*Lycopersicon esculentum*) cultivars were transformed with genes that encode bacterial chitinolytic enzymes (i.e., endochitinase and chitobiosidase) from *Streptomyces albidoflavus*. Transgenic tomato plants producing these enzymes were found to have enhanced resistance to cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), consistently reducing the growth rates of larvae. Mortality was significantly increased in two of three feeding trials. Ingestion of endochitinase and chitobiosidase not only affected development of larval *T. ni* from neonate to ultimate instar, but they also caused mortality and decreased insect weight when exposure began during the third instar. The results of this study provide some insight into the mode of action of the chitinolytic enzymes, by supporting the hypothesis that ingested chitinolytic enzymes damage the chitin component of the peritrophic envelope, leading to increased permeability. The size of marker molecules (FITC-dextrans) that permeated the peritrophic envelopes of *T. ni* feeding on transgenic plants were 50% larger than those permeating the peritrophic envelopes of *T. ni* feeding on the control plants. Further research is needed to more clearly identify the sites and modes of action of these chitinolytic enzymes, and the potential for synergy between these enzymes and pathogens, allelochemicals, and other environmental factors.

Introduction

Chitin (β-1,4-linked *N*-acetyl-β-D-glucosamine) is the principal structural component of the linings of the digestive tract of insects. In the foregut and hindgut, chitin is found in a cuticular lining, whereas in the midgut, chitin is present in peritrophic membranes (collectively termed the peritrophic envelope). Peritrophic membranes are composed of a meshwork of chitin microfibrils onto which a matrix of glyco-proteins, proteoglycans, and proteins is complexed (Peters, 1992). Unlike the cuticle lining the foregut and hindgut, the peritrophic envelope is continuously secreted, either from the entire midgut epithelium (Type 1) or from a specialized ring of cells in the anterior midgut (Type 2). The protective functions of the peritrophic envelope include shielding the mi-

crovilli from abrasive food particles, preventing the entry of ingested pathogenic microbes, acting as an antioxidant, and ultrafiltration of certain allelochemicals (Abedi & Brown, 1961; Brandt et al., 1978; Sudha & Muthu, 1988; Sieber et al., 1991; Barbehenn & Martin, 1992, 1995). In addition, the peritrophic envelope is believed to compartmentalize digestive enzymes by dividing the midgut into endo- and ectoperitrophic spaces, thereby improving the efficiency of digestion (Terra & Ferreira, 1981; Eguchi et al., 1982; Miller & Lehane, 1990; Zhu et al., 1991; Peters, 1992).

Chitinolytic enzymes catalyze the hydrolysis of chitin at the C1-C4 bond between *N*-acetylglucosamine units (Bielka et al., 1984; Sahai & Manocha, 1993). There are three types of chitinolytic enzymes: (I) *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), which cleaves terminal *N*-acetylglucosamine units from

chitin, (II) 1,4-β-D-chitobiosidase (EC 3.2.1.14) ('exochitinase'), which cleaves terminal dimeric units from chitin, and (III) endochitinase (EC 3.2.1.14), which randomly cleaves chitin internally. All three types of chitinolytic enzymes are synthesized by organisms that contain chitin, such as insects, crustaceans, yeasts, and fungi (Chen, 1987), as well as by organisms that do not contain chitin, such as bacteria, higher plants, fish, and humans (Benmouna et al., 1986; Grisley & Boyle, 1990; Collinge et al., 1993; Hollak et al., 1994).

Chitinolytic enzymes produced by pathogenic microbes and nematodes enable them to gain entry into insect hosts through the exoskeleton or internally through the peritrophic envelope (Tanada & Kaya, 1993). Several researchers have explored the potential use of chitinolytic enzymes for insect pest control. Brandt et al. (1978) showed that chitinolytic enzymes can degrade the peritrophic envelope of *Orgyia pseudotsugata, in vitro*. Chitinolytic enzymes from *Streptomyces griseus* added to the blood meal of the mosquito, *Anopheles freeborni*, were found to prevent the peritrophic envelope from forming (Shahabuddin & Kaslow, 1993; Shahabuddin et al., 1993). Supplementation of an inoculum of entomopathogens with microbial chitinolytic enzymes enhances the effectiveness of the insect pathogens (El-Sayed et al., 1989; Sampsom & Gooday, 1998). In addition, an endochitinase from *Serratia marcescens* produced as a recombinant protein in *Escherichia coli* was found to perforate the peritrophic envelope of *Spodoptera littoralis* larvae *in vitro* (Regev et al., 1996).

The chitinous lining of the digestive tract of herbivorous insects is continuously exposed to ingested chitinolytic enzymes from plant tissues. However, there is no indication that plant chitinolytic enzymes have a detrimental effect on larval Lepidoptera (Kramer et al., 1997). This lack of biological activity against caterpillars is likely due to the acidic pH optima of these enzymes (Broadway et al., 1998), and the alkaline midguts of caterpillars (Berenbaum, 1980; Mishra & Sen-Sarma, 1987; Gringorten et al., 1993). Most fungal and bacterial chitinolytic enzymes also have optimal activity in an acidic environment. However, Broadway et al. (1995) isolated a strain of *Streptomyces albidoflavus* that secretes endochitinolytic enzymes and chitobiosidases that are active over a broad range of pH (4–10). Ingestion of this mixture of chitinolytic enzymes in artificial diets significantly reduced the growth and development of *Trichoplusia ni* (Lepidoptera: Noctuidae) (Broadway et al., 1995). In this study, tomato plants (*Lycopersicon esculentum*) were transformed with endochitinase and chitobiosidase genes isolated from *S. albidoflavus*. These plants were evaluated for their effect on the growth and development of larval *T. ni*. In addition, we tested the hypothesis that ingested chitinolytic enzymes damage the peritrophic envelope, as indicated by an increase in its permeability to marker molecules.

Materials and methods

Gene cloning

The endochitinase and chitobiosidase genes isolated from a genomic DNA library from *S. Albidoflavus* were engineered in the binary vector pBin 19 and were identified as pS.a-endochitniase and pS.a- chitobiosidase. A full-length cDNA clone of endochitinase and chitobiosidase in a Bluescript SK$^-$ vector (Stratagene) were PCR amplified using a specific forward primer with a *Nco* restriction enzyme site and a reverse primer with a *Xba* site. Primers used for the PCR amplification of the endochitinase gene were: Ep1- (5′-GATCAACCATGGGCTACTTCACCGAGTGGGGC-3′) and Ep2-(5′-TTCGTCTAGACTAGCGGAGGCCGGAGTC-3′). Primers used for the PCR amplification of the chitobiosidase gene were: BpEXP1 (5′-TTCCACCATGGCCGCGCTACCGGCCCAC-3′) and BpEXP2 (5′-GATCCGTCTAGATTACGGCAGGTCGTCGAC-3′).

The amplified cDNA was clone into vector pBI525 (provided by Dr W. G. W. Kurz, National Research Council of Canada's Plant Biotechnology Institute) digested previously with *Nco* and *Xba*. The chitobiosidase gene has a *Nco* restriction enzyme site as part of the DNA sequence. Therefore, it was necessary to digest the PCR fragment first with *Xba* and then to perform a partial digestion with *Nco*. After confirming by sequencing that both genes were in frame, the insert of the expression cassette from pBI525 was excised with *Eco*R I and *Hind* III, and cloned in the plasmid pBin19. Following PCR to identify colonies containing recombinant plasmids with inserts of the expected sizes, plasmid DNA was isolated and gene insertion confirmed by restriction enzyme analysis.

For the construction of the double-gene construct (pS.a- endochitinase-chitobiosidase) pS.a- endochitinase was digested with *Eco*R I. Dephosphorylation of linearized pS.a-endochitinase was performed. The chitobiosidase gene that was cloned in the plasmid pBI525 was amplified using the forward primer

pUCf (5′-TGGAATTCCGGCTCGTATGTTGTG-3′), and the reverse primer pUCr (5′-TTCCCAGTCACGA CGTTGTAAA-3′), both designed with an *Eco*R I restriction enzyme site. The samples were then digested with *Eco*RI. After digestion, cohesive-termini chitobiosidase gene was added to a ligation reaction containing pS.a-endochitinase dephosphorylated plasmid. Competent cells were transformed with the ligation solution. Identification of colonies containing recombinant plasmids was performed.

Plant transformation

The plasmid product identified as pS.a- endochitinase-chitobiosidase, was used for the transformation of tomato plants. Transformation and regeneration of tomato were accomplished by a modification of Mc-Cormick et al. (1986) reported by Xue et al. (1994). *Agrobacterium tumefaciens* LBA 4404 containing the construct pS.a- endochitinase-chitobiosidase was used. The selection of the transformed tissue was done on a selection medium containing 50 mg/L of kanamycin and 500 mg/L of carbenicillin. Four tomato cultivars were used for the transformation: UC82B, Better Boy VFN, Beefmaster VFN (BM), and Geneva 80. Following transformation and regeneration, the tomato plants were transferred to soil, maintained in the greenhouse and allowed to grow until they produced fruit. The seeds were collected for further use.

Identification and characterization of transformed plants

Presence of the nptII gene in the plant genome. The *npt*II gene in the transformation vectors confers resistance to the antibiotic kanamycin and was used as a selective marker in the transgenic plants. Greenhouse-grown tomato plants with four leaves were used for DNA extraction. DNA was isolated as described by Cheung et al. (1993). Polymerase chain reaction (PCR) amplification was performed on genomic DNA to determine the presence of the *npt*II marker gene in first and second generation tomato lines. As controls, the plasmid T-DNA (region of the transformation vector transferred from *A. tumefaciens* to the plant) containing the *npt*II gene was amplified. The PCR cycle consisted of 94 °C × 5 min [1 cycle], 94 °C × 30 s, 67 °C × 30 s, 72 °C × 1 min [30 cycles], and 72 °C × 5 min [1 cycle]. PCR amplification was used to confirm the presence of the *npt*II marker gene prior to the use of tomato plants for chitinase assays

or insect bioassays. Enzyme-linked immuno-sorbant assays (ELISA) were used to test the expression of the *npt*II marker gene in newly-transformed tomato lines (T0 lines). All T0 tomato plants that were NPTII positive, based on ELISA analysis, were grown in the greenhouse until they produced fruit, and the seeds were collected and planted. The germinated seeds (T1 plants) were characterized by PCR amplification of the *npt*II gene. The plants that were identified as *npt*II positive were transplanted to soil in pots, maintained in the greenhouse, and Southern blot analysis was performed, as described below.

Presence of the chitinase transgenes in the plant. Southern blot analyses were performed on *npt*II-positive transgenic tomato lines to determine (1) if the chitinase transgenes were inserted in the plant genome, and (2) the number of copies per genome. DNA from first generation tomato lines grown in a greenhouse was isolated using the technique described by Fulton et al. (1995). Fresh young leaf material, weighing 50 mg, was immersed in 200 μl of extraction buffer. Tissue was then ground with an electric motor-driven Kontes pestle for about 30 s. An extra 550 μl of extraction buffer was added, and the samples were vortexed. The lysate was incubated for 1 h at 60 °C. After incubation, the sample was extracted twice with chloroform:isoamyl alcohol and precipitated with isopropanol. Precipitated DNA was resuspended in 25 μl of water. RNAase was added (final concentration 20 μg ml^{-1}), and the sample was incubated (37 °C, 30 min). For restriction enzyme digestion of genomic DNA, 10–15 μg of genomic DNA was incubated (overnight, 37 °C) with 100 U of the restriction enzyme *Hin*dIII and 5 μl of Multi-Core buffer (Promega, Madison, WI) in a final reaction volume of 50 μl.

Southern blots analyses: DNA (plasmid or genomic) was separated in 1% agarose gel, denatured by immersing the gel in denaturing solution (0.5 N NaOH, 1.5 M NaCl), neutralized with a solution containing 1 M Tris-HCl and 1.5 M NaCl (pH 8). DNA samples were transferred overnight by capillary transfer to a nylon membrane (Geneplus, NEN Research Products, Boston, MA) using 10× buffer (1.5 M NaCl, 150 mM sodium citrate, pH 7). The membrane was washed with 5× buffer containing 0.1% sodium dodecyl sulfate (SDS) for 3 min at room temperature. The DNA was fixed to the membrane by exposing it to UV light, and the membrane was loaded in a hybridization tube. The membranes were blocked by

incubating samples (2 h, 60–65 °C) in 15 ml blocking solution (10% dextran sulfate, 1% SDS, and 1 M NaCl). Random oligonucleotide primers were used for the labeling reactions of the probes (Sambrook et al., 1989). Templates for the radioactive probes included: (1) the T-DNA in the pS.a-endochitinase-chitobiosidase, (2) the cloned chitobiosidase inserted in the plasmid pBluescript II SK$^-$ (Stratagene, La Jolla, CA), and (3) the cloned endochitinase gene from pS.a endochitinase. The endochitinase and chitobiosidase were excised with *Nco* and *Xba* from the plasmids, separated in a 1.5% agarose gel, cut from the gel under illumination with UV (330 nm), and purified from the gel using the Prep-A-Gene kit (BioRad, Richmond, VA). Each template (50 μl containing 50 ng of pS.a-endochitinase-chitobiosidase for the labeling of the T-DNA or the endochitinase or chitobiosidase fragments) was denatured by boiling for 10 min, and immediately put on ice. An aliquot (45 μl) of the template was added to a solution containing 11 μl LS buffer, 4 μl bovine serum albumin, 1 U of Klenow fragment (Promega), and 5 μl of ^{32}P α-CTP (NEN Research Products, Boston, MA), and incubated at 37 °C for 30 min. After incubation, the probes were separated from unincorporated dCTPs by using Sephadex G-50 Nick Spin Columns (Amersham Pharmacia Biotech, Piscataway, NJ), and 1 μl of the sample was quantified for radioactive labeling with a scintillation counter. Microcentrifuge tubes containing the radioactive probe were boiled (10 min), cooled on ice, and added to the hybridization tube for overnight incubation. Membrane development was performed [according to the manufacturer's protocol for Genescreen Plus TM (NEN Research Products)]. Film was placed on top of the membrane, and exposed for 2 to 10 days, depending of the signal intensity, at −80 °C before developing.

Chitinolytic enzymes in planta. The presence of transgenic chitobiosidase and endochitinase in the progeny of transgenic tomato in the greenhouse was determined by Western blot analysis. The buffer-soluble proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis (Sambrook et al., 1989) using polyclonal antibodies raised against the bacterial chitinolytic enzymes.

Proteins were isolated from leaf samples (50 mg) (Gegenheimer, 1990) by homogenization in 150 μl of Bradley buffer (50 mM Tris pH 7.5, 10 mM KCl, 20% glycerol, 0.4 M sucrose, 5 mM MgCl$_2$, supplemented with 10 mM 2-mercaptoethanol, 10 mM pefablock and 10 mM chymostatin prior to use). Samples were homogenized with a motor-driven Kontes pestle (30 s), and centrifuged (16 000 × g, 5 min, room temperature). An aliquot (50 μl) of supernatant was mixed with 50 μl of Laenly buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue). The samples were boiled for 5 min and centrifuged (16 000 × g, 1 min) The protein separation was done in a vertical electrophoresis system (Mini-Protean II cell, Bio-Rad). An aliquot (25 μl) of the sample was loaded on an SDS-polyacrylamide gel (12% polyacrylamide resolving gel and 5% polyacrylamide stacking gel) (Hames & Rickwood, 1990). The gels were run in running buffer (25 mM Tris, 192 mM glycine, pH 8.3, 0.1% SDS) at 100 V and 10–40 mA. Proteins were blotted from the gel using the Mini Trans-Blot cell (Bio-Rad) and Immobilon-P transfer membranes with 0.45 UM (Millipore, Bedford, MA) in electrophoresis transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, PH 8.3) at 100 V for 1 h (Hames & Rickwood, 1990). After blotting, the membranes were transferred to a blocking solution [PBS (pH 7), 0.05% Tween 20, 10% non-fat dry milk], and agitated for 1 h. They were incubated overnight at 4 °C in a solution of primary rabbit anti-semipurified *S. albidoflavus* endochitinase (or chitobiosidase) extract at a concentration of 1:100 antibody (1 mg ml^{-1}):blocking solution. The membranes were washed five times with the washing solution [PBS (pH 7), 0.05% Tween] (5 min per wash), incubated in the blocking solution plus the secondary antibody anti-rabbit Ig horseradish peroxidase (Amersham Life Science, Little Chalfont, Buckinghamshire, England) (1:1000 dilution) for 1 h on a rocker, and then washed five times with the washing solution. The membranes were exposed to chemiluminescent detection using emission chemiluminescence luminol Western blotting (ECL) following the standard protocol (Amersham Life Science). The film was then placed on top of the membrane, and exposed for 5–30 min before developing.

Chitinase activity in planta. Plants that expressed high chitinase activity were identified by enzyme assays using methyl umbelliferyl (MU) substrates. Leaf samples (10 to 15 mg) were homogenized in 50 μl of buffer (0.1% SDS, 0.1% Triton X-100, 10 mM Na$_2$ EDTA, 10 mM 2-mercaptoethanol, 100 mM sodium acetate buffer, adjusted to pH 4.8) per mg of tissue (as described above) and stored on ice. Samples were

centrifuged ($16\,000 \times g$, 4 °C, 5 min) and an aliquot (100 μl) of the supernatant solution was mixed with 40 μl of substrate (1 mg methyl umbelliferyl N,N,N triacetyl chitotriose or methyl umbelliferyl N,N,N triacetyl chitobiose in 3.5 ml of 100 mM sodium acetate buffer, pH 5). After incubation at 28 °C for 30, 60, 90, 120 or 150 min the reaction was stopped by mixing an aliquot (20 μl) of the reaction mixture with 180 μl of 0.2 M Na_2CO_3. Fluorescence was measured with a CytoFluorII scanner using the CytoFluorII program (Excitation: 360/40 nm; Emission: 460/40 nm; Gain: 70). A standard curve was calculated based on the fluorescence of 4-methylumbelliferone (0, 20, 40, 60, 80, 100, 150, 200 mM) dissolved in 0.2 M Na_2CO_3.

Protein concentration was determined for each sample using the Bio-Rad protein assay, based on the Bradford dye-binding procedure (Bio-Rad). Bovine serum albumin standards, dissolved in umbelliferyl assay buffer, were prepared at concentrations of 0, 20, 50, 80, and 120 μg. The reagent was prepared by diluting the Bio-Rad protein assay dye reagent 1:5 with deionized water. Aliquots (200 μl) of the diluted reagent were transferred to a 96 well microtiter plate, and 2 μl of standard or leaf extract was added. After mixing, the plates were read in a SLT Spectra ELISA plate reader at 620 nm. Activity slopes (nM MU /min) were determined for each sample, and the value of nM MU/min was expressed relative to the amount of protein present (nM MU min^{-1} μg^{-1} protein).

Chitinase activity in different leaves of the plant. Expression of chitinolytic enzymes in different leaves of the tomato plant was examined to determine which leaflets would be used for the insect bioassays. Each experiment included ten transgenic (BmB1–1) plants and ten control plants. All plants were two months old with seven to eight leaves on the main stem, and the leaves were numbered 1 to 8 from young to old. Three leaflets were removed from each plant: one leaflet from a fully-developed leaf from the upper part of plant (leaf 1 or 2), one leaflet from a leaf from the middle part of the plant (leaf 3 or 4), and one leaflet from a leaf from the bottom part of the plant (leaf 5 or 6). The levels of endochitinase and chitobiosidase were quantified by enzymatic activity assays using methyl umbelliferyl substrates.

Effect of transgenic plants on insects

Insects. Eggs of *T. ni* were provided by Dr W. L. Roelofs (NY State Agricultural Experiment Station, Cornell University, Geneva, New York). Larval *T. ni* were reared on a high wheat germ meridian diet (Webb & Shelton, 1988). Insects were maintained at 26 °C and 60–70% relative humidity.

Choice of tomato cultivar. The tomato cultivar used in insect bioassays was chosen based on two factors: high growth and survivorship of *T. ni* on control plants and sufficient seed production by transgenic plants. After confirming that the T-DNA sequence, or at least part of it, was present in some lines from the transformed tomato cultivars, and that the chitobiosidase gene was present in the cultivars Beefmaster VFN and Better Boy VFN, we selected one cultivar to be used for further evaluations. For that purpose, nontransgenic control plants of UC82B ($N = 15$), Better Boy VFN ($N = 15$), and Beefmaster VFN ($N = 13$) that were 30 to 40 days old (four to five pairs of leaves) were infested with *T. ni*. The second or third leaf of each plant was enclosed in a clear plastic cage (20–30 cm long, 10 cm diameter) containing two neonate *T. ni* larvae. The plants infested with the insects were maintained in the greenhouse at 25–35 °C and 16 h photoperiod. Larvae were allowed to develop until they reached the final instar, at which time larval survivorship and final weight were measured. UC82B produced the lowest percent insects recovered and the lowest mean larval weight (i.e., 13%, 23 ± 20 mg) compared to the other two cultivars. Better Boy VFN resulted in the greatest recovery of insects (i.e., 73%, 70 ± 51 mg), but only one line of transgenic plants was obtained and the number of seeds produced was low. Therefore, this cultivar was not evaluated further. Beefmaster resulted in a high recovery of large insects (i.e., 70%, 78 ± 54 mg), eight different putative transgenic lines that were *nptII* positive, and a large number of seeds were produced by some of the T0 plants. Based on this information, Beefmaster was the cultivar selected for future evaluations.

Initially the seeds of three lines of Beefmaster VFN were germinated, and labeled lines A1, B1, and H3. Line A1 was discarded because it was *nptII* negative. The level of endochitinase and chitobiosidase enzymatic activity was evaluated in lines B1 and H3. ANOVA indicated that line H3 did not differ statistically from the control in chitinase activity (i.e., endochitinase activity F=0.34, P=0.575; chitobiosidase activity F= 3.0, P=0.12). However, the level of enzyme activity for line B1 was significantly higher than the control for endochitinase activity (F=21.66, P<0.001) and chitobiosidase activity

(F=10.98, P=0.006). Line B1 was used for further evaluation and insect bioassays.

Effect of transgenic plants on larval performance. Prior to the insect bioassays, the enzyme activity assay using umbelliferyl substrates was performed to determine the level of endochitinase and chitobiosidase expression in the transgenic plants. The effect of transgenic tomato plants on neonate larvae was evaluated using two sets of plants. Set 1 consisted of first generation transgenic progeny from BmB1 (18 transgenic and 15 control plants). Two insect bioassays were performed using this set of plants. The first bioassay was performed when the plants were two months old and a second bioassay was performed when the plants were three months old. Set 2 consisted of second generation transgenic progeny, grown from seeds from first generation BmB1 plant #7. One insect bioassay was performed on two-month-old control ($n = 20$) and transgenic ($n = 30$) plants. Two leaflets from a young tomato leaf (control or transgenic plants) were cut at the base of the petiole with a razor blade. To minimize the induction of a wound response in the plants resulting from removal of the leaflets from the leaves, we avoided damage to the major veins (Ryan, 1974). To maintain leaf turgor, the petiole of each leaflet was inserted into 5 ml of solid 2% agar (w/v) in a 100 ml glass jar with magenta B-caps. One insect was placed on each leaflet, and the leaflets were replaced daily. The jars containing the insects feeding on the leaflet were maintained in a growth chamber at 25±2 °C with a 16-h photoperiod. Larvae were weighed upon reaching the final instar, and insects were monitored daily for developmental changes until they reached the adult stage.

The effect of transgenic tomato plants on third-instar *T. ni* was evaluated using two-month-old plants grown from seeds from two first-generation BmB1 plants. This experiment included ten transgenic and ten control plants, and was performed as described above.

Effect of ingested transgenic tomato on peritrophic envelope permeability. Larvae were reared on artificial diet until they reached the third instar. The third-instar larvae were transferred at random to excised leaflets from transgenic tomato plants (two-month-old first-generation transgenic progeny from BmB1 set I plants; $N = 10$) or control plants ($N = 10$), as described above. Larvae were allowed to feed for ten days, and leaflets were replaced as they were consumed. After ten days, larvae were individually chilled (−20°C, 2 min) and dissected under a dissecting microscope. The entire midgut was removed from each larva and placed in a dish of incubating solution (Barbehenn & Martin, 1995). Each gut (eight controls and eight treatments) was ligated with size 6-0 suture, and the open end of the gut preparation was tied over the needle tip of a 10 μl Hamilton syringe held in a micromanipulator. An aliquot (1 μl) of 2000 nominal MW fluorescein isothiocyanate labeled dextran (FITC-dextran, Sigma Chemical, St. Louis, MO) (80 mg ml^{-1}) was injected into the lumen. The gut preparation was removed from the needle tip while keeping tension on the suture to prevent the leakage of the gut contents. Gut preparations were maintained under the surface of the incubating solution at all times. Because of the potentially delicate state of the peritrophic envelope in larvae fed transgenic foliage, no hole was cut through the gut wall. Previous work showed that the gut wall does not affect the size distribution of FITC-dextrans permeating the intact gut (Barbehenn & Martin, 1995). Gut preparations were rinsed three times in beakers of incubating solution, and placed in separate aliquots (1.5 ml) of incubating solution for 1.5 h. The entire volume of incubating solutions was lyophilized and stored in a desiccator (2 °C, 0.5 months). The size distribution of the FITC-dextran that diffused through each gut preparation was determined by high-performance size-exclusion chromatography (Barbehenn & Martin, 1997). Samples were resolubilized in 0.3 ml double-distilled water and filtered (0.45 u, Gelman GHP Acrodisc). Aliquots (30 ul) were separated on a Synchrom GPC-300 HPSEC column (250 × 4.5 mm) with guard column, using a 0.5 M sodium phosphate (pH 7) mobile phase at 1 ml min^{-1} (30 oC). FITC-dextran was detected with a Shimadzu RF 535 fluorescence detector (492 nm excitation, 516 nm emmission), and peak area and retention time was quantified with a Shimadzu C-R4A Chromatopac integrator. FITC-dextran standards (4000–2 000 000 MW; Sigma) were used to make standard curves. The experiment was repeated as described above, with the following exceptions: 3rd–5th-instar larvae were used, 133 mg ml^{-1} FITC-dextran was injected, 12 control and 12 treatment larvae were analyzed, and lyophilized samples were stored three months.

One-way analysis of variance (ANOVA) using the Minitab 10.51 xtra Power software (Minitab Inc, State College, PA) was used to compare the levels of endochitinase and chitobiosidase activity in transgenic and control plants, and to compare insect biomass and survival on these plants. Two-way ANOVA (treatment × experiment) was used to compare the mean diameters of FITC-dextrans permeating the gut preparations from control and treatment larvae. Post-hoc multiple comparisons of treatment means was made with least significant difference (LSD) tests.

Results

Presence of transgenes in tomato leaves. The tomato Southern blot, using a T-DNA probe (synthesized from the construct pS.a-endochitinase-chitobiosidase), showed that the cultivar Beefmaster VFN line B1 ($N = 3$) had five copies of the T-DNA, while the cultivar Better Boy VFN line C1 had three copies of the T-DNA (Figure 1). In the cultivar UC82B, two lines showed two copies of the T-DNA, and another line showed one copy (data not shown). No regenerants were obtained from the line Geneva 80. The tomato Southern blot using a chitobiosidase probe in the cultivar Better Boy VFN (one line) showed that all the plants analyzed had two copies of the chitobiosidase gene, while the cultivar Beefmaster VFN line B1 had three copies of the gene present in the two plants analyzed (data not shown).

Chitinolytic activity in different parts of the tomato plant. Endochitinase activity in leaflets from the upper, middle, and bottom portion of the foliage of two-month-old transgenic and control tomato plants was determined. The relative amount of endochitinase (i.e., the mean endochitinase activity in the transgenic plants divided by the mean endochitinase activity in the controls) produced by the leaflets in the upper part of the foliage of the plants was significantly greater than the relative amount of endochitinase in the leaflets from the middle and bottom part of the foliage (upper leaflets vs. middle leaflets F=7.58, P=0.019, and upper leaflets vs. bottom leaflets F=8.92, P=0.014) (Figure 2). No differences were found between the leaflets in the middle and the bottom of the plant (F=0.09, P=0.769). Similar results were obtained with respect to the amount of chitobiosidase (data not
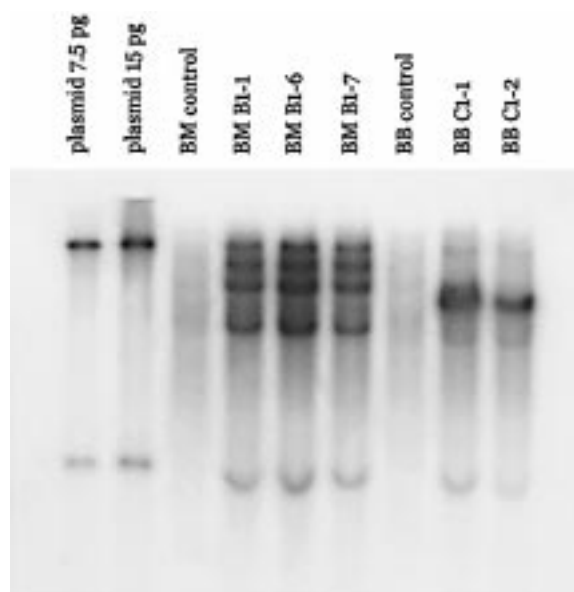

Figure 1. Tomato cultivar Beefmaster (BM) and Better Boy (BB) Southern blot using a T-DNA probe synthesized from the construct pS.a-endochitinase-chitobiosidase. Tomato cultivar Beefmaster-transgenic line B1 shows five copies of the genes and tomato cultivar Better Boy - transgenic line C1 shows three copies of the genes. Lanes were loaded as follow: lane 1 and lane 2 with 7.5 pg and 15 pg respectively of pS.a-endochitinase-chitobiosidase digested with *Hin*dIII. All the other lanes were loaded with 15 μg of DNA digested with *Hin*dIII. Lane 3 (BM control) contains a Beefmaster non-transgenic plant. Lane 4 (BM B1-1), lane 5 (BM B1-6), and lane 6 (BM B1-7) correspond to three different samples from the same Beefmaster B1 line. Lane 7 (BB control) contains a Better Boy non-transgenic sample. Lane 8 (BB C1-1), and lane 9 (BB C1-2), correspond to two different samples from the same Better Boy C1 line.

shown). Based on these results the leaflets from the upper part of the foliage (leaves 1 and 2) of the tomato plants were used for the insect bioassays.

Expression of transgenic chitinolytic enzymes in tomato leaves used in the insect bioassays. Upper leaves of two-month-old first-generation transgenic plants produced significantly higher endochitinase and chitobiosidase activities than did control plants (F=10.23, P=0.003 and F=5.16, P=0.03, respectively) (Figures 3A and 3B). Further verification that elevated chitinolytic activity in the transgenic plants resulted from the expression of the transgenes (pS.a-endochitinase-chitobiosidase) was made by using Western blots. Using anti-endochitinase antibodies, a band of 39.5 kDa indicated the presence of this protein in first-generation transgenic plants (Figure 4). Using anti-chitobiosidase antibodies, a band
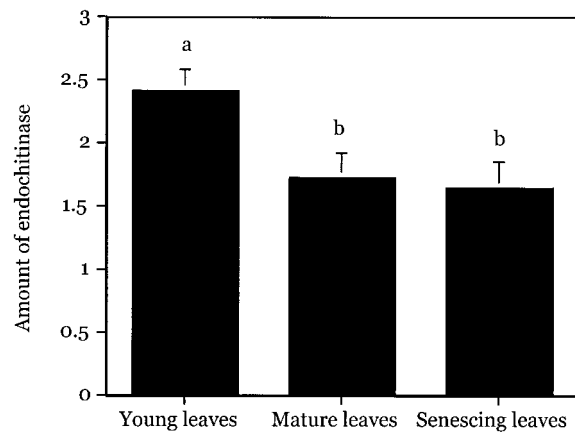
Figure 2. Relative endochitinase activity (mean endochitinase activity in the transgenic plants divided by the mean endochitinase activity in the controls) in T1 BmB1 tomato plants. The bars represent the relative amount of endochitinase activity in (1) Leaflets from the upper part of the plant (young leaves), (2) Leaflets from the middle part of the plant (mature leaves) and (3) Leaflets from the bottom part of the plant (senescing leaves). Vertical lines indicate $\pm$ 1 SE. Columns associated with the same letter are not significantly different.

of 31.6 kDa indicated the presence of this protein in second generation transgenic plants (Figure 5).

Effect of transgenic tomato on T. ni*: neonate larvae to adulthood.* Ingestion of leaves from two months old T1 transgenic plants significantly increased mortality in larval *T. ni*. Mortality in the controls was 26% compared to 61% in the insects feeding on the transgenic leaves (Figure 6; bioassay 1). Those insects that survived the ingestion of leaves from transgenic plant were significantly smaller than larvae feeding on control plants (F= 8.33, P=0.001) (Figure 7; bioassay 1). The mean weight of the insects feeding on foliage from control plants (N =20) was twice the mean weight of the insects feeding on the transgenics (N =16). Using the same group of plants when they were three months old resulted in similar effects on the insects. The ingestion of the leaves from the transgenic plants significantly increased mortality in larval *T. ni*. Mortality in the controls was 47% compared to 78% in the insects feeding on the transgenic leaves (Figure 6; bioassay 2). In the insects that survived the ingestion of leaves from the transgenic plant, a significant reduction in larval weight was observed compared with the insects feeding on the control plants (F = 6.77, P=0.016) (Figure 7; bioassay 2). The mean weight of insects feeding on the control plants (N = 17) was 1.3 fold higher than the mean weight of the insects feeding
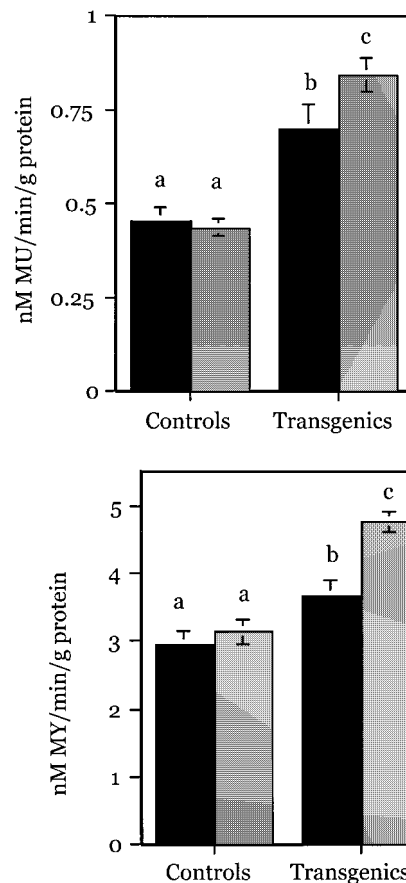

Figure 3. (A) Endochitinase activity in control and transgenic tomato plants. Black bars represent the enzymatic activity in plants (T1) used for bioassays 1 and 2. Gray bars represent the enzymatic activity in plants (T2) used for bioassay 3. Vertical lines indicate $\pm$ 1 SE. Columns associated with the same letter are not significantly different. (B) Chitobiosidase activity in control and transgenic tomato plants. Bars are labeled as described above.

on the transgenic plants (N = 8). The rate of development was monitored for those larvae that survived the feeding study. Significant differences were observed in the two groups of insects (controls vs. transgenic) with respect to the number of larvae that reach adulthood. In the controls, 34% of insects reached adulthood, while only 6% of the insects feeding on transgenic foliage matured.

The ingestion of leaves from the T2 transgenic plants when they were two months old significantly reduced the weight of larval *T. ni* (F = 26.63, P<0.001) (Figure 7; bioassay 3). The mean weight of larvae feeding on the controls) (N = 33) was 1.6 fold higher than the mean weight of larvae feeding on the transgenic foliage (N = 53). After determination of their weight, the insects were monitored for rate of de-
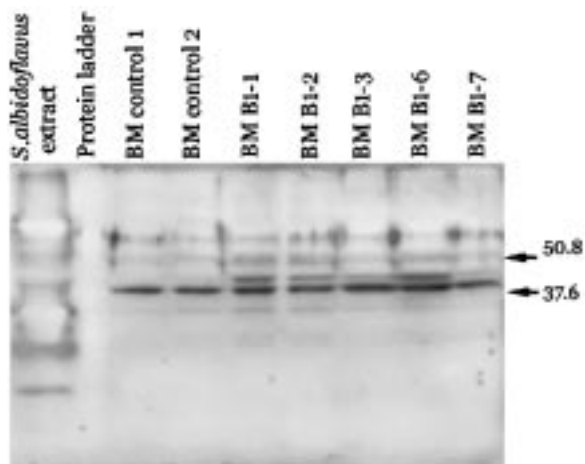
Figure 4. Western blot showing expression of the *S. albidoflavus* endochitinase transgene in the progeny of transgenic tomato cultivar Beefmaster (BM). A band of 39.5 kDa indicates the presence of the protein. Lanes were loaded as follows: lane 1 (*S. albiflavus* extract) with 50 μg of chitinolytic extract from *S. albidoflavus*. Lane 2 with Benchmarkprestained protein ladder. Lanes 3 and 4 (BM control) with extracts from Beefmaster non-transgenic plants, not expressing the chitinase transgene. Lane 5 (BMB1-1), lane 6 (BMB1-2), lane 7 (BMB1-3), lane 8 (BMB1-6), and lane 9 (BMB1-9) with extracts from five different plants from the line B1, endochitinase positive progeny.
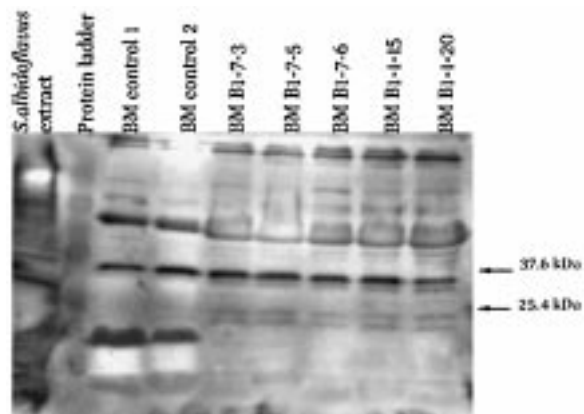

Figure 5. Western blot showing expression of the *S. albidoflavus* chitobiosidase transgene in the progeny (T2) of transgenic tomato cultivar Beefmaster (BM). A band of 31.6 kDa indicates the presence of the protein. Lanes were loaded as follows: lane 1 (*S. albiflavus* extract) with 50 μg of chitinolytic extract from *S. albidoflavus*. Lane 2 with Benchmark prestained protein ladder. Lanes 3 and 4 (BM control) with extracts from Beefmaster non-transgenic plants, not expressing the chitinase transgene. Lane 5 (BM B1-7-3), lane 6 (BM B1-7-5), lane 7 (BM B1-7-6), lane 8 (BM B1-1-15), and lane 9 (BM B1-1-20) with extract from five different plants from the progeny of B1 (T2), chitobiosidase positive progeny.
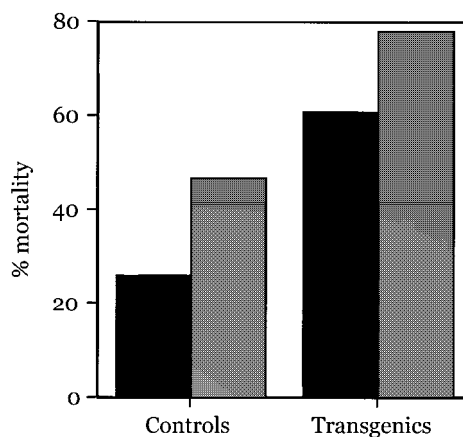

Figure 6. Effect of transgenic tomato plants on mortality of larval *Trichoplusia ni*. Bars represent the percent mortality of larvae feeding on leaves from control Beefmaster, or transgenic Beefmaster (T1 line BmB1), after insects feeding on the control plants reached the fifth instar. Black bars represent the percent mortality in bioassay 1, and gray bars represent the percent mortality in bioassay 2.

velopment until they reached adulthood. The insects in the control group ($N = 25$) reached the adult stage in 23 days, while the insects in the transgenic group ($N = 40$) reached the adult stage in 27 days. This difference in developmental time was significant ($F = 73.51$, $P < 0.001$).

Effect of transgenic tomato on T. ni*: third to fifth instar.* Final weights of fifth-instar larvae fed on transgenic tomato foliage averaged 60% of the weights of larvae fed control foliage (0.07 ± 0.01 g and 0.12 ± 0.01 g, respectively) ($F = 8.06$, $P = 0.013$). Mortality of larvae fed transgenic leaves was 40%, whereas no mortality was observed for insects feeding on the control leaves.

Effect of ingested transgenic tomato on peritrophic envelope permeability. The mean diameter of polydisperse FITC-dextrans permeating the peritrophic envelop in larvae fed transgenic plants (24.0 ± 2.6 nm) was approximately 1.5 times the size of FITC-dextrans that permeated the pores in peritrophic membranes of larvae fed control foliage (16.4 ± 1.4 nm) ($F = 6.80$, $P = 0.013$). No differences were found between experiments ($F = 0.427$, $P = 0.517$), nor was there a significant interaction between experiment and treatment ($F = 0.848$, $P = 0.363$).
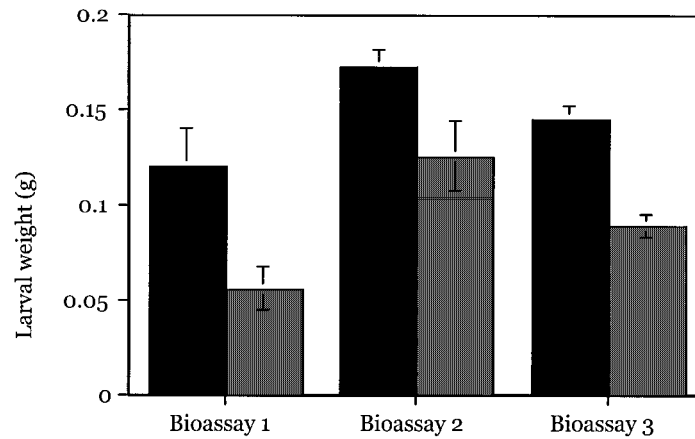
Figure 7. Effect of tomato transgenic plants on weight of larval *Trichoplusia ni*. Black bars represent the mean weight of insects feeding control Beefmaster plants. Gray bars represent the mean weight of insects feeding on Beefmaster transgenic plants after insects feeding on the control leaflets reached the fifth instar. Bioassay 1 and 2 correspond to insects feeding on T1 BMB1 plants when they were two and three month old respectively. Bioassay 3: correspond to insects feeding on two month old plants (T2 BmB1 1-7). Vertical lines indicate ± 1 SE.

Discussion

In this study tomato cultivars were transformed with two genes that encode bacterial chitinolytic enzymes (endochitinase and chitobiosidase). Unlike endogenous plant chitinolytic enzymes and some chitinolytic enzymes previously inserted into plant genomes, the bacterial chitinolytic enzymes used in this study are active at the high pH of caterpillar midgut lumens (Broadway et al., 1998). Transgenic tomato plants producing these enzymes were found to have enhanced resistance to *T. ni* larvae, consistently reducing the growth rates of *T. ni*. In addition, mortality was significantly increased in two of three feeding trials. The results of this study are similar to those found in previous works on artificial diets containing chitinolytic enzymes: the primary impact on *T. ni* is a reduction in larval weight and development, and increase in mortality (Broadway et al., 1998). In addition, ingestion of the plants expressing those enzymes not only affected larval *T. ni* following ingestion from neonate to ultimate instar, but they also caused mortality and decreased insect weight when exposure began during the third instar.

These results also suggest that tomato transgenic plants are causing an effect on *T. ni* larvae similar to the one caused by the ingestion of diet supplemented with 0.25 to 0.5% (w/v) mixture of chitinolytic enzymes (Broadway et al., 1998). Possibly, an increase in the levels of chitinase activity in transgenic plants will have a greater effect on larval Lepidoptera. Future efforts to improve the resistance of chitinase-producing transgenic crops against insects should include enhancing chitinase gene expression, and/or the use of the additional chitinolytic enzymes present on the mixture of enzymes from *S. albidoflavus* (Broadway et al., 1998).

The results of this study support the hypothesis that ingested chitinolytic enzymes from *S. albidoflavus* damage the chitin component of the peritrophic envelope, leading to increased permeability. The size of marker molecules (FITC-dextrans) that permeated the peritrophic envelopes of *T. ni* feeding on transgenic plants were 50% larger (24 nm) than those permeating the peritrophic envelopes of *T. ni* feeding on the control plants (16.4 nm). However, the peritrophic envelopes of larvae were largely intact, as indicated by the restriction of movement of FITC-dextran particles with an average diameter larger than 40.1 to 41.6 nm. Further work is needed to determine whether there were any pores large enough to admit pathogens, such as baculoviruses (60 × 300 nm).

The focus of previous research that has targeted the peritrophic envelope has been on (1) reducing peritrophic envelope permeability, (2) inhibiting chitin or protein synthesis (Clarke et al., 1977; Cohen, 1993; Zimmerman & Peters, 1987), or (3) disrupting peritrophic membrane structure directly (Derksen & Granados, 1988; Peng et al., 1999). The strategy taken in this study is representative of the third approach. An increase in the pore sizes in peritrophic membranes may make the peritrophic envelope a less effective barrier, increasing the susceptibility of insects to pathogens and toxic compounds (Brandt et al., 1978;

Sieber et al., 1991). Recent work suggests that the peritrophic envelope has limited protective capabilities against most ingested allelochemicals (Barbehen & Martin, 1997). However, in addition, the peritrophic envelope is believed to compartmentalize digestive enzymes by dividing the midgut into endo- and ectoperitrophic spaces, thus improving the efficiency of digestion (Eguchi et al., 1982; Miller & Lehane, 1990; Peters, 1992; Terra & Ferreira, 1981; Zhu et al., 1991). Therefore, a marked increase in the permeability of the peritrophic envelope might decrease the efficiency of the digestive process. Our results indicate that ingestion of transgenic bacterial chitinolytic enzymes enhance resistance of tomato plants against larval *T. ni*, and reduce the growth, development and survival of larval *T. ni* feeding on the transgenic foliage. However, we have not demonstrated that the increased pore size of the peritrophic envelope was directly linked to the negative impact on larval *T. ni*. Further work is needed to examine this link, and to determine whether synergistic effects occur when insects are exposed to ingested chitinolytic enzymes along with factors, such as pathogens or allelochemicals.

Acknowledgements

References

Abedi, Z. H. & A. W. Brown, 1961. Peritrophic membrane as a vehicle for DDT and DDE excretion in *Aedes aegypti* larvae. Annals of the Entomological Society of America 54: 539–542.

Barbehenn, R. V. & M. M. Martin, 1992. The protective role of the peritrophic membrane in the tannin-tolerant larvae of *Orgyia leucostigma* (Lepidoptera). Journal of Insect Physiology 38: 973–980.

Barbehenn, R. V. & M. M. Martin, 1995. Peritrophic envelope permeability in herbivorous insects. Journal of Insect Physiology 41: 303–311.

Barbehenn, R. V. & M. M. Martin, 1997. Permeability of the peritrophic envelopes of herbirous insects to dextran sulfate: a test of the polyanion exclusion hypothesis. Journal of Insect Physiology 43: 243–249.

Benmouna, H., M. F. Jaspar-Versali, C. Toussaint & C. Jeuniaux, 1986. A comparative study of chitinase activity in digestive tract of *Serranum cabrilla* and *Serranus scriba*. Biochemical Systematics and Ecology 14: 435–437.

Berenbaum, M., 1980. Adaptive significance of midgut pH in larval Lepidoptera. American Naturalist 115: 138–146.

Bielka, H., H. B. F Dixon, P. Karlson, C. Liebecq, N. Sharon, E. J. Van Lenten, S. F. Velick, J. F. G. Vliegenthart, E. C. Webb, A. Cornish-Brown, K. Loening, G. P. Moss, & J. Reedijk, 1984. In: E. C. Webb (ed), Enzyme Nomenclature. Academic Press, New York, 308 pp.

Brandt, C. R., M. J. Adang & K. D. Spence, 1978. The peritrophic membrane: Ultrastructural analysis and function as a mechanical barrier to microbial infection in *Orgyia pseudotsugata*. Journal of Invertebrate Pathology 32: 12–24.

Broadway, R. M., D. L. Williams, W. C. Kain, G. E. Harman, M. Lorito & D. P. Labeda, 1995. Partial characterization of chitinolytic enzymes from *Streptomyces albidoflavus*. Journal of Applied Microbiology 20: 271–276.

Broadway, R. M., C. Gongora, W. C. Kain, J. P. Sanderson, J. A. Monroy, K. C. Bennet, J. B. Warner & M. P. Hoffmann, 1998. Novel Chitinolytic enzymes with biological activity against herbivorous insects. Journal of Chemical Ecology 24: 985–998.

Chen, A. C., 1987. Chitin metabolism. Archives of Insect Biochemistry and Physiology 6: 267–277.

Cheung, W. Y., N. Hubert & B. S. Laundry, 1993. A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and other PCR analysis. PCR Methods Applied 3: 69–70.

Clarke, L., G. H. R. Temple & J. F. V. Vincent, 1977. The effects of a chitin inhibitor dimilin on the production of peritrophic membrane in the locust locusta-migratoria. Journal of Insect Phisiology 23: 241–246.

Cohen, E., 1993. Chitin synthesis and degradation as targets for pesticide action. Archives of Insect Biochemistry and Physiology 22: 245–261.

Collinge, D. B., K. M. Kragh, J. D. Mikkelsen, K. K. Nielsen, U. Rasmussen & K. Vad, 1993. Plant Chitinases. Plant Journal 3: 31–40.

Derksen, A. C. G. & R. R. Granados, 1988. Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology 167: 242–259.

Eguchi, M., A. Iwamoto & K. Yamauchi, 1982. Interrelation of proteases from the midgut lumen, epithelia and peritrophic membrane of the silkworm, *Bombyx mori* L. Comparative Biochemistry and Physiology 72A: 359–363.

El-Sayed, G. N., T. A. Coudron, C. M. Ignoffo & G. Riba, 1989. Chitinolytic activity and virulence associated with native and mutant isolates of an entomopathogenic fungus, *Nomuraea raleyi*. Journal of Invertebrate Pathology 54: 394–403.

Fulton, T. M., J. Chunwongse & S. D. Tansksley, 1995. Micropep protocole for extraction of DNA from tomato and other herbaceous plants. Plant Molecular Biology Report 13: 207–209.

Gegenheimer, P., 1990. Preparation of extracts from plants. Methods in Enzymology 182: 174–193.

Gringorten, J. L., D. N. Crawford & W. R. Harvey, 1993. High pH in the ectoperitrophic space of the larval lepidopteran midgut. Journal of Experimental Botany 183: 353–359.

Grisley, M. S. & P. R. Boyle, 1990. Chitinase, a new enzyme in octopus saliva. Comparative Biochemistry and Physiology 95B: 311–316.

Hames, B. D. & D. Rickwood, 1990. Gel Electrophoresis of Proteins: A Practical Approach. IRL Press, New York.

Hollak, C. E., S. Van Welly, M. H. Van Oers & J. M. Aert, 1994. Marked elevation of plasma chitotriosidase activity. Journal of Clinical Investigation 93: 1288–1292.

Kramer, K. J., S. Muthukrishnan, L. Johnson & F. White, 1997. Chitinase for insect control. In: N. Carozzi & M. Koziel (eds), Advances in Insect Control: the Role of Transgenic Plants. Taylor & Francis, London, pp. 185–193.

204

McCormick, S., J. Niedermeyer, J. Fry, A. Barnason, R. Horsch & R. Fraley, 1986. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Report 5: 81–84.

Miller, N. & M. J. Lehane, 1990. *In vitro* perfusion studies on the peritrophic membrane of the tsetse fly *Glossina morsitans morsitans* (Diptera, Glossinidae). Journal of Insect Physiology 36: 813–818.

Mishra, S. C. & P. K. Sen-Sarma, 1987. pH trends in the gut of xylophagous insects and their adaptive significance. Material und Organismen 22: 311–319.

Peng, J., J. Zhong & R. R. Granados, 1999. A baculovirus enhancin alters the permeability of a mucosal midgut peritrophic matrix from lepidopteran larvae. Journal of Insect Physiology 45: 159–166.

Peters, W., 1992. Peritrophic Membranes. Zoophysiology Series. Vol. 30, Springer-Verlag, Berlin. 238 pp.

Regev, A., M. Keller, N. Strizhov, B. Sneh, E. Prudovsky, I. Chet, I. Ginzberg, K. Z. Koncz, C. Koncz, J. Schell & A. Zilberstein, 1996. Synergistic activity of a *Bacillus thuringiensis* delta-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Applied and Environmental Microbiology 62: 3581–3586.

Ryan, C. A., 1974. Assay and biochemical properties of the proteinase inhibitor-inducing factor, a wound hormone. Plant Physiology 54: 328–332.

Sahai, A. S. & M. S. Manocha, 1993. Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiological Reviews 11: 317–338.

Sambrook, J., E. F. Fritsch & T. Maniatis, 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sampsom, M. N. & G. W. Gooday, 1998. Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. Microbiology 144: 2189–2194.

Shahabuddin, M. & D. C. Kaslow, 1993. Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. Parasitology Today 9: 252–255.

Shahabuddin, M., T. Toyoshima, M. Aikawa & D. C. Kaslow, 1993. Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. Procedures of the National Academy of Science of the United States of America 90: 4266–4270.

Sieber, K. P., M. Huber, D. Kaslow, S. M. Banks, T. Motomi, M. Aikawa & L. H. Miller, 1991. The peritrophic membrane as a barrier: its penetration by *Plasmodium gallinaceum* and the effect of a monoclonal antibody to ookinetes. Experimental Parasitology 72: 145–156.

Sudha, P. M. & S. P. Muthu, 1988. Damage to the midgut epithelium caused by food in the absence of peritrophic membrane. Current Science, India 57: 624–625.

Tanada, Y. & H. Kaya, 1993. Fungal Infections. In: Insect Pathology. Academic Press, San Diego, CA, pp. 318–366.

Terra, W. R. & C. Ferreira, 1981. The physiological role of the peritrophic membrane and trehalase: digestive enzymes in the midgut and excreta of starved larvae of *Rhinchosciara*. Journal of Insect Physiology 27: 325–331.

Webb, S. E. & A. M. Shelton, 1988. Laboratory rearing of the imported cabbageworm. NY Food Life Science. Bull 122.

Xue, B., C. Gonsalves, R. Providenti, J. L. Slightom, M. Fuchs & D. Gonsalves, 1994. Development of transgenic tomato expressing a high level of resistance to cucumber mosaic virus strains of subgroups I and II. Plant Disease 78: 1038–1041.

Zhu, Z., L. Gern & A. Aeschlimann, 1991. The peritrophic membrane of *Ixodes ricinus*. Parasitology Research 77: 635–641.