Comparative Biochemistry and Physiology, Part B 168 (2014) 45–52

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Variant vicilins from a resistant *Vigna unguiculata* lineage (IT81D-1053) accumulate inside *Callosobruchus maculatus* larval midgut epithelium

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A R T I C L E I N F O

Article history:
Received 11 September 2013
Received in revised form 2 November 2013
Accepted 5 November 2013
Available online 9 November 2013

Keywords:
Protein absorption
Endocytosis
Antibiosis
Insect midgut
Vicilin

A B S T R A C T

It has been demonstrated that variant vicilins are the main resistance factor of cowpea seeds (*Vigna unguiculata*) against attack by the cowpea beetle *Callosobruchus maculatus*. There is evidence that the toxic properties of these storage proteins may be related to their interaction with glycoproteins and other microvillar membrane constituents along the digestive tract of the larvae. New findings have shown that following interaction with the microvilli, the vicilins are absorbed across the intestinal epithelium and thus reach the internal environment of the larvae. In the present paper we studied the insecticidal activity of the variant vicilins purified from a resistant cowpea variety (IT81D-1053). Bioassays showed that the seeds of this genotype affected larval growth, causing developmental retardation and 100% mortality. By feeding *C. maculatus* larvae on susceptible and IT81D-1053 derived vicilins (FITC labelled or unlabelled), followed by fluorescence and immunogold cytolocalization, we were able to demonstrate that both susceptible and variant forms are internalized in the midgut cells and migrate inside vesicular structures from the apex to the basal portion of the enterocytes. However, when larvae were fed with the labelled vicilins for 24 h and then returned to a control diet, the concentration of the variant form remained relatively high, suggesting that variant vicilins are not removed from the cells at the same rate as the non-variant vicilins. We suggest that the toxic effects of variant vicilins on midgut cells involve the binding of these proteins to the cell surface followed by internalization and interference with the normal physiology of the enterocytes, thereby affecting larval development in vivo.

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1. Introduction

Cowpea (*Vigna unguiculata*) (Fabaceae) seeds provide a major source of cheap and high-quality dietary protein, particularly in tropical regions in Africa and Central and South America. However, cultivated cowpeas are subject to severe post-harvest losses caused by larvae of bean-feeding beetles (Coleoptera: Chrysomelidae: Bruchinae), also known as bruchids. One of the most important pests of stored cowpea is the cowpea beetle (*Callosobruchus maculatus*). Due to the adaptations developed by this stored-bean pest, seed destruction is often so high that the pulse grains become unsuitable for human consumption and unviable for replanting or commercialization.

The cowpea beetle has become a cosmopolitan pest of Old World leguminous pulses, particularly seeds of the genus *Vigna*, e.g. *Vigna unguiculata* and *Vigna angularis*. Female bruchids cement their eggs to the surface of seeds and approximately six days later, the first-instar larvae hatch and burrow through the seed tegument to reach the cotyledons. Larval development (four instars) and pupation are completed entirely within a single host seed. Adults emerge from the seeds through a “pupal window” leaving behind a typical hole and are able to mate and oviposit within a question of few hours (Dick and Credland, 1984). This species is easy to breed and handle, and laboratory colonies can be maintained in conditions similar to their anthropic

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http://dx.doi.org/10.1016/j.cbpb.2013.11.001

niche. As *C. maculatus* larvae cannot change their food source (the seed chosen by the female), larval development will only be successful if the larvae are capable of dealing with the defensive compounds present in the seeds.

In the 1970's, screening of the cowpea germplasm collection at the International Institute of Tropical Agriculture (Ibadan, Nigeria), allowed the identification of the first genotype highly resistant to *C. maculatus*, the cultivar TVu 2027 (Gatehouse et al., 1979), from which several other lineages such as IT81D-1032, IT81D-1053, IT81D-1045, and IT81D-1064, that show different levels of resistance to the cowpea beetle were bred. The observed antibiosis was firstly attributed to relatively high levels of trypsin inhibitors (Gatehouse et al., 1979), but subsequently it was demonstrated that the resistance was in fact caused by the presence of variant vicilins (Macedo et al., 1993). The defensive role of variant vicilins against the cowpea beetle was first reported from the genotype IT81D-1045, that is derived from the cultivar TVu 2027 and is also resistant to *C. maculatus* (Macedo et al., 1993). The mechanism of action of the variant vicilins appears to be correlated to their resistance to proteolysis by digestive peptidases and their binding affinity to *C. maculatus* midgut epithelial cell surfaces (Firmino et al., 1996; Sales et al., 2001).

In addition to the evidence indicating that cowpea vicilins interact with constituents on the surface of *C. maculatus* larval midgut epithelium, the systemic distribution of vicilins in larvae has also been investigated. Uchôa et al. (2006) demonstrated that vicilins were absorbed intact through the midgut of *C. maculatus* larvae, and where subsequently detected in the haemolymph and internal organs such as fat body and malphigian tubules. The absorbed vicilin molecules were detected in adult females and males after emergence and were also deposited in the eggs following copulation (Souza et al., 2010; Alexandre et al., 2011).

In light of these new findings, questions concerning the mechanism of toxicity of variant vicilins were further investigated. With the aim of understanding the putative toxic effects of variant vicilins to *C. maculatus* larvae, we traced the fate of labelled vicilins, purified from susceptible and resistant varieties, in whole larvae with emphasis on their localization in the midgut tissue. Evidence reported in this study showed that ingestion of variant vicilins leads to the accumulation of vesicles that persist for longer time periods in the cytoplasm of enterocytes when compared with ingestion of vicilins from a susceptible cultivar.

2. Material and methods

2.1. Rearing of insects

The colony of *C. maculatus* used in this work was initiated with animals originally supplied by Dr. J.H.R. Santos, Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Brazil. Stock cultures of this species have been maintained continuously since 1978. Insects were reared on *V. unguiculata* seeds (commercial cultivar known as *Fradinho*) in natural photoperiod, 29 ± 1 °C and relative humidity of 65 ± 5%.

Two *V. unguiculata* genotypes were utilized in this study to feed *C. maculatus* larvae. The susceptible commercial cultivar *Fradinho* and the resistant lineage IT81D-1053, that was bred from TVu 2027, the first recorded cultivar resistant to *C. maculatus*.

2.2. Development of *C. maculatus* on different cowpea seeds

To measure the development of *C. maculatus* on the commercial cultivar and on IT81D-1053, five replicates of 10 seeds of each cultivar were used. Two fertilized females (2–3 days old) were placed in glass bottles each containing 10 cowpea seeds and left to oviposit for 24 h. After this period, the insects were removed and the eggs laid in excess of one were removed. The number of emerging adults was used to calculate the percentage survival to adult emergence (S). The days on which the adults emerged were also recorded to estimate the mean development period (T). Larval development was also photographed for comparative means.

2.3. Vicilin purification and antibody production

Vicilins were purified from *C. maculatus* susceptible (Epace 10) and resistant (IT81D-1053) seeds employing the procedure developed by Macedo et al. (1993). Ground seed flour was extracted with 50 mM borate buffer (pH 8.0) for 30 min at room temperature, centrifuged (30 min at 8000 *g*, 5 °C) and the soluble proteins were fractionated by ammonium sulphate precipitation. The 70–90% saturation fraction was dialysed against distilled water, freeze-dried and chromatographed on a DEAE-Sepharose column (2 × 20 cm) equilibrated with 50 mM Tris–HCl (pH 8.0) and eluted with a NaCl gradient (0–1 M) in the same buffer. The vicilin-rich fractions were then loaded onto a Sephacryl S-400 column (2.5 × 70 cm) in 0.1 M Tris–HCl, 0.25 M NaCl (pH 8.0). Fractions containing vicilins were dialysed against distilled water and freeze-dried.

Antiserum against purified Epace 10 vicilin was prepared by immunisation of rabbits according to standard methods. Purified antiserum was obtained from the Serviço de Produção de Anticorpos Monoclonais e Policlonais, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

2.4. Covalent conjugation of vicilin with FITC (fluorescein isothiocyanate)

FITC was covalently coupled to either susceptible (Epace 10) or resistant (IT81D-1053) vicilins. FITC (50 mg in 1 mL anhydrous DMSO) was immediately dissolved in 0.75 M bicarbonate buffer (pH 9.5) before use. Following addition of FITC to a ratio of 1 mg per mg of vicilin, the tube was wrapped in foil; incubated and rotated at room temperature for 1 h. Un-reacted FITC was removed by dialysis against distilled water. The resulting solution was freeze-dried.

2.5. Feeding *C. maculatus* larvae using artificial seeds or artificial galleries made in natural seeds

The technique described in this paper was based on a technique published by Souza et al. (2010). Actively feeding *C. maculatus* larvae were transferred at the beginning of the first or the fourth instar to gelatin capsules (BDL empty capsules trading Co.) containing the different mixtures to be tested. To verify the fate of the labelled vicilins in *C. maculatus* larvae, the FITC–vicilin complexes (both susceptible and resistant) were mixed with cowpea flour (Fradinho) at the concentration of 0.5% (w/w). The flour was inserted and compacted inside empty gelatin capsules with the aid of spatulas and glass rods. Larvae removed from seeds of *V. unguiculata* were transferred to a cavity produced in the compacted mass of flour in one half of the gelatin capsule, at a ratio of two larvae per capsule. The capsules were then carefully mounted in order to permit the feeding movements of the larvae. Care not to injure the larvae and a depression that simulates a gallery work to provide an environment that allows the larvae to feed and complete their life cycle.

After the feeding period (24 h), larvae were removed and processed for light or electron microscopy or they were transferred back to control gelatin capsules containing only cowpea flour, where they were kept for an additional 48 h. Following this period on the control diet, the larvae were removed from the capsules and prepared for light or electron microscopy. Larvae fed inside capsules containing only cowpea flour were used as controls to evaluate autoflourescence of the internal organs. The equivalent of 30 larvae for each treatment was used and the representative results were chosen for documentation and publication.

2.6. Preparation of samples from insects

In order to visualize and document the presence of labelled vicilins using fluorescence microscopy, whole larvae were placed in a drop of

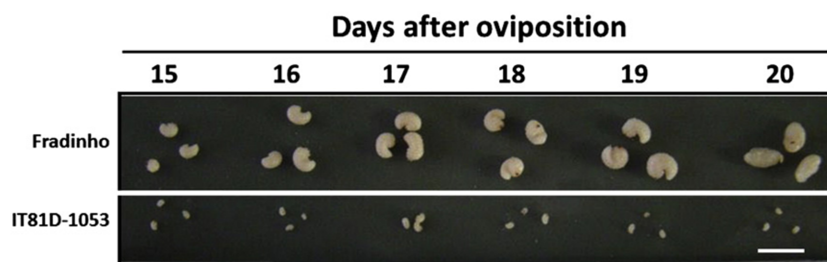

Fig. 1. Inhibitory effect of seeds of the resistant lineage IT81D-1053 on the growth of *Callosobruchus maculatus* larvae. Upper panel, insects reared on susceptible seeds (*Fradinho*). Bottom panel, insects reared on resistant seeds (IT81D-1053). Bar: 0.5 cm.

distilled water, dried with filter paper and incubated in a increasing sucrose grade beginning at 5% sucrose for 3 h at room temperature, then transferred to 15% sucrose for 3 h at room temperature and finally 18 h in 30% sucrose at 4 °C. Following the sucrose baths, the larvae were placed in their respective blocks and filled with the resin (Tissue-Tek®) for 1 h at a ratio of 2 larvae per block until completely frozen. At the end of this period, sections were cut in a Leica Cryostat (Model CM1850) to a thickness of 7 μm and placed on glass slides for viewing and documentation using a fluorescence microscope (Leica DMI6000 B Microscope). FITC–vicilin complexes were excited with the 488 nm laser line, and the emitted fluorescence was detected between 500 and 560 nm. In order to compare different experimental conditions (i.e., larvae fed with susceptible or variant labelled vicilins) the image acquisitions were always performed with the same microscope settings. The equivalent of 30 larvae for each treatment was used and the representative results were chosen for documentation.

In order to test the specificity of the anti-vicilin polyclonal antibody, pooled midgut tissue samples were extracted from fourth instar larvae after 16–17 days of development in susceptible seeds (ten animals per preparation). Only actively feeding larvae with food filling the gut tracts were chosen for dissection. Pre-chilled larvae were rinsed with saline, dried with filter paper and the whole gut was removed. Pooled midgut tissues, after being freed from luminal contents by rinsing in cold 150 mM NaCl, were homogenized in the same saline using a hand-held Potter–Elvehjem homogeniser immersed in ice. Midgut tissue homogenates were centrifuged at 15,000 g for 15 min at 4 °C and the supernatant (soluble fraction) was collected and the sediment (membrane fraction) was homogenized in distilled water with the aid of a Potter–Elvehjem homogeniser. Both soluble (containing free vicilin molecules) and membrane fractions (containing membrane-bound vicilin molecules) were used for electrophoresis and Western blotting.

2.7. Protein determination

Protein concentration was determined according to the method of Bradford (1976) using ovalbumin as a standard.

2.8. Electrophoresis and Western blotting

Proteins were separated by SDS polyacrylamide gel electrophoresis (Laemmli, 1970). Samples (20 μg protein) were prepared by adding 4× SDS sample buffer and boiled for 5 min prior to loading on gels. Gels were run at a constant voltage of 150 V and either stained using Coomassie blue dye (0.05% [w/v] Coomassie blue in 7% [v/v] glacial acetic acid; 40% [v/v] methanol) followed by de-staining (19% [v/v] glacial acetic acid, 40% [v/v] methanol), or transferred to nitrocellulose membranes using a BioRad Trans-blot semi dry transfer cell, according to the manufacturer's recommendations.

Immunoblotting was performed using the polyclonal antibody against purified susceptible vicilin as described above. The primary antibody was used at a dilution of 1:20,000 and the secondary antibody was diluted 1:2000. Binding was visualised using a horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Richmond, CA, USA) according to manufacturer's instructions.

2.9. Immunocytochemistry

Callosobruchus maculatus larvae (2nd instar, 12 days after oviposition or 4th instar, 18 days after oviposition) reared on Fradinho cowpea seeds were used for immunocytochemical analysis. The material was fixed in 4% paraformaldehyde/0.3% glutaraldehyde in 0.1 sodium cacodylate buffer, pH 7.2, for 2 h with alternate shaking and vacuum treatments. The material was then washed in sodium cacodylate buffer (3 times, 10 min each), dehydrated in a series of ethanol/water mixtures at 30, 50, 70, 90 and 100% ethanol and then embedded in hydrophilic LR White acrylic resin. Microtome sections (70 nm) were prepared for immunolabelling using anti-vicilin antibodies (1:500). The distribution of bound anti-vicilin in the sections was determined using secondary antibodies conjugated with colloidal gold (15 nm). Control sections were incubated without reaction with the primary antibody.

3. Results

3.1. Development of C. maculatus on seeds of the cultivar IT81D-1053

The development of our strain of *C. maculatus* on seeds of the susceptible commercial cowpea cultivar used in this study showed high

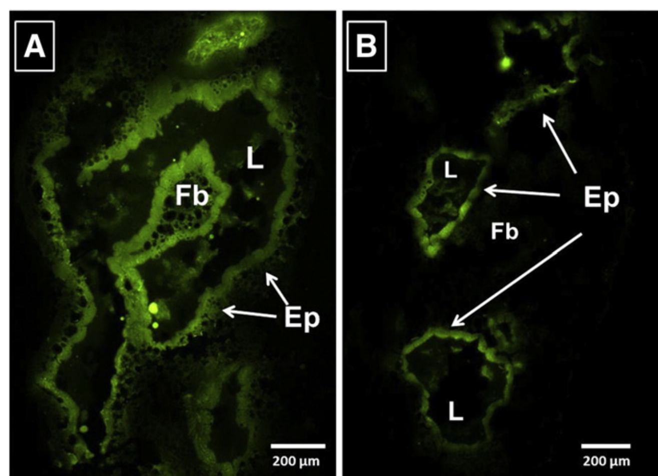

Fig. 2. Fluorescence microscopy localization of FITC labelled vicilins in the internal organs of larval *Callosobruchus maculatus*. First instar larvae were fed with a mixture of 0.5% FITC–vicilin/cowpea flour for 24 h. Control animals were fed with unlabelled susceptible vicilins incorporated in the cowpea flour, however the results were not shown here as fluorescence was minimal. Plate A: typical fluorescence micrograph obtained from a larva fed on susceptible-vicilin–FITC. Plate B: typical fluorescence micrograph obtained from a larva fed on IT81D-1053–vicilin–FITC. Note fluorescence in the midgut lumen (L), in the midgut epithelium (Ep) (arrows) and in the fat body lobules (Fb). FITC, fluorescein isothiocyanate.

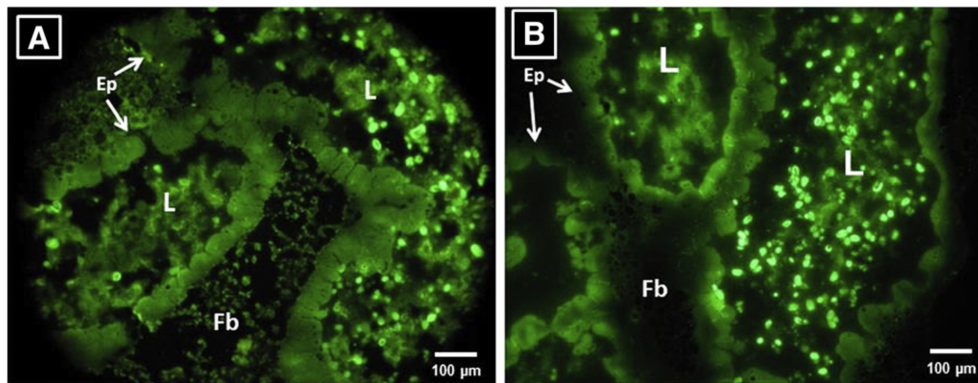

Fig. 3. Fluorescence microscopy localization of FITC-labelled vicilins in fourth instar *Callosobruchus maculatus* larvae. Animals were fed with a mixture of 0.5% FITC–vicilin/cowpea flour for 24 h. Plate A: typical fluorescence micrograph obtained from larvae fed on susceptible-vicilin–FITC. Plate B: typical fluorescence micrograph obtained from larvae fed on IT81D-1053–vicilin–FITC. Note: fluorescence in the midgut lumen (L), in the midgut epithelium (Ep) (arrows) and in the fat body lobules (Fb). FITC, fluorescein isothiocyanate.

survival rates with 100% adult emergence and a mean development time from egg to adult of 29.7 $\pm$ 0.5 days. Conversely, the larvae developing on the lineage IT81D-1053 did not complete their development, even after 60 days following oviposition, showing that this cultivar, genetically bred from TVu 2027, is completely resistant to *C. maculatus*. The larvae remained alive 60 days after oviposition, but did not complete their development to adults during that period. Inspection of the galleries showed that larvae had fed, but did not develop normally.

A comparison between the larval development on the two types of seeds showed that while larvae increased continuously in size and moulted normally when feeding on the susceptible cultivar, larvae fed on IT81D-1053 seeds showed no increase in size or any evidence of moulting (Fig. 1, bottom panel).

3.2. Tracking the fate of the IT81D-1053 variant vicilins using fluorescence microscopy

Fluorescence microscopy demonstrated that both FITC-labelled susceptible and resistant vicilins were internalized in *C. maculatus* larval midgut cells (Fig. 2) and they can also be detected in fat body cells (Fig. 3). Analysis of the fluorescent images at high magnification showed the uptake of labelled-vicilins in vesicular structures of enterocyte cytoplasm (Fig. 4). Experiments involving pulse feeding of larvae on labelled vicilins from susceptible or resistant beans for 24 h, followed by two days of feeding on control diet, was performed to compare the uptake of vicilins in the midgut cells and their deposition in fat body cells. As shown in Fig. 5, the fluorescence intensity of the internalized labelled resistant vicilins was much higher than the fluorescence observed for the susceptible vicilins.

3.3. Immuno-electron localization of vicilins in larval C. maculatus midgut cells

Immuno-electron cytochemical experiments were performed using midguts dissected from larvae fed on both susceptible and resistant cowpea seeds. A specific antiserum was used in these experiments (Fig. 6). The antibody recognized the vicilin subunits and also recognized the same subunits in samples of intestinal epithelia from larvae fed on Fradinho grains. In the sample containing only the particulate fraction, the subunits were recognized while in the soluble fraction in addition to the vicilin subunit bands, lower molecular mass bands were also observed. In resin sections, vicilin immunolabelling was observed on circular structures in the lumen of the midgut epithelium, resembling vesicular protein structures ingested from the seed cotyledon (Fig. 7C). These structures were also observed in the microvillar membranes (Figs. 7A,B,C and 8A,B). Both forms of vicilins were internalized at the apical membrane of columnar cells and were found in vesicular structures localized in the cell apex (Figs. 7A,B and 8A,B). Labelling was also seen close to the basal region of the enterocytes (Fig. 8C) and associated with the basal labyrinth of the midgut cells (Figs. 7C and 8C).

4. Discussion

Vicilins (7S storage globulins) have received much attention due to their antimicrobial and insecticidal properties, as well as other biological activities (Bäumlein et al., 1995; Shutov et al., 1995; Braun et al., 1996; Chung et al., 1997; Gomes et al., 1997; Shutov et al., 1998; Wang et al., 2001; Mota et al., 2003; Ng, 2004; Manners, 2007; Marcus et al., 2008; Paes et al., 2008). When orally administered in artificial diets, vicilins cause detrimental effects to non-adapted insects, including binding to peritrophic membranes leading to reduced growth and increased mortality when compared to control diets (Mota et al., 2003; Paes et al., 2008). It has been demonstrated that variant vicilins of cowpea seeds are the main resistance factor present in some African cowpea genotypes, protecting the seeds against attack by the bean beetle *C. maculatus* (Macedo et al., 1993; Sales et al., 2001). The available evidence suggest that the toxic effects of the variant vicilins, purified from the resistant cowpea variety IT81D-1045, towards larval *C. maculatus* are linked to resistance of these vicilins to hydrolysis (Sales et al., 1992), chitin-binding activity (Sales et al., 1996) and binding to the midgut microvilli leading to antibiosis, putatively due to interference in the physiology of the midgut cells (Firmino et al., 1996; Sales

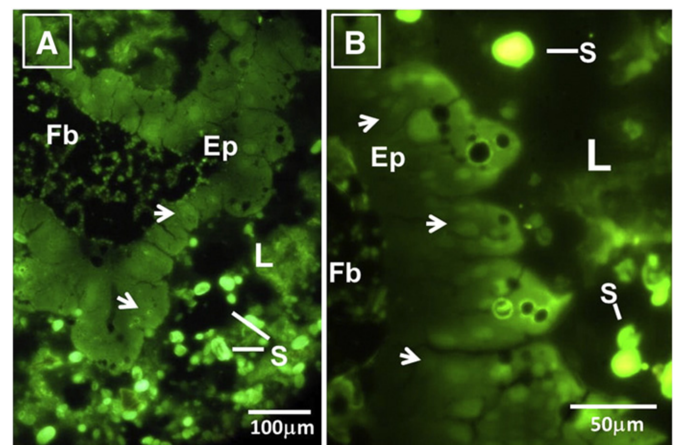

Fig. 4. Fluorescence microscopy demonstrating vicilin localization in the cytoplasm of *Callosobruchus maculatus* larvae midgut cells. Fourth instar larvae were fed a mixture of cowpea flour plus 0.5% FITC-labelled IT81D-1053 vicilin for 24 h. Note: fluorescence in the midgut lumen (L), in the midgut epithelium (Ep) and in the fat body (Fb). It was possible to see highly fluorescent starch granules (S) in the midgut lumen. In the enterocyte cytoplasm several fluorescent vesicular structures can be seen (arrowheads). Panel A: 10× objective; panel B: 40× objective.

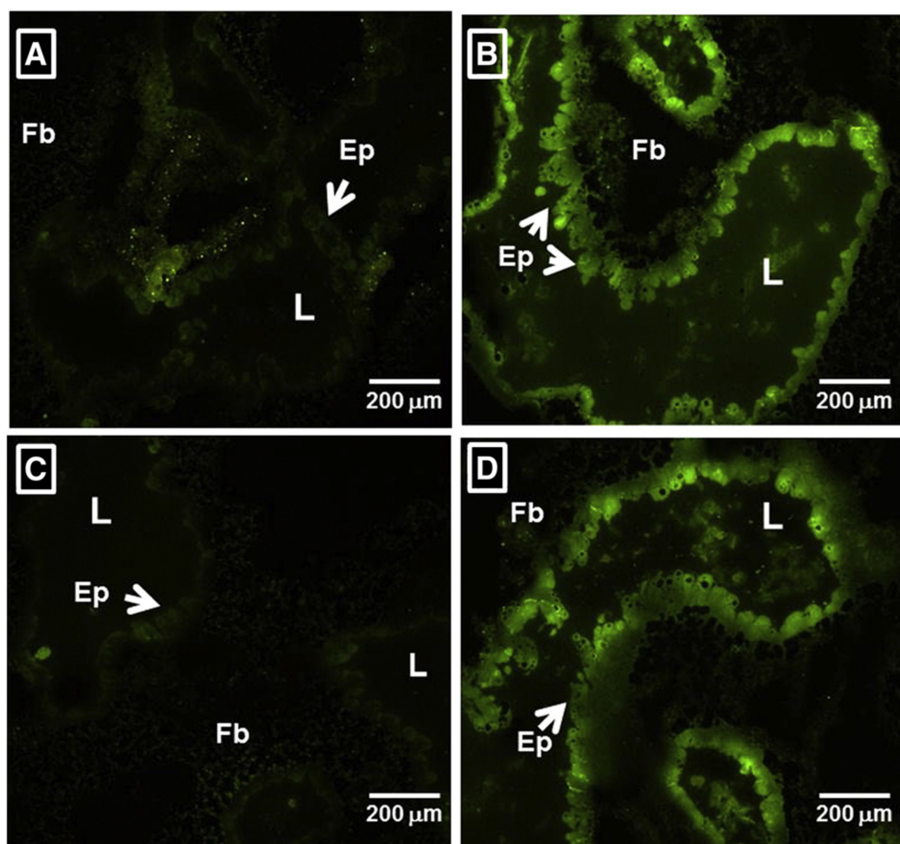

Fig. 5. Fluorescence microscopy detection of FITC–vicilins in whole larval *Callosobruchus maculatus* after a 24 h feeding period on labelled-susceptible or-IT81D-1053 vicilins, followed by a 48 h feeding period on control diet. Fourth instar larvae were fed with mixtures of 0.5% FITC–vicilins for 24 h inside gelatine capsules. After this period, the larvae were transferred to gelatine capsules containing cowpea flour only and left to feed for an additional 48 h. Panels A and C: susceptible-vicilin fed animals; B and D: IT81D-1053–vicilin fed animals. Note: higher fluorescence in the midgut epithelial cells of IT81D-1053–vicilin fed animals than in susceptible-vicilin fed ones (Ep, arrowheads). L, midgut lumen; Fb, fat body lobules; FITC, fluorescein isothiocyanate.

et al., 2001). Sales et al. (2001) demonstrated through immunostaining experiments that variant vicilins from the IT81D-1045 variety bound with higher affinity to midgut cell membrane preparations than susceptible vicilins. Furthermore, immuno-electron localization of vicilins showed that the luminal gut wall surface was strongly labelled. However, careful inspections of the published micrographs revealed that vicilins derived from the resistant variety IT81D-1045 can be seen not only on the surface of intestinal epithelial cells, but also within these cells and also in fat body cells (see Fig. 5 in Sales et al., 2001). More recent findings have demonstrated that vicilin molecules are absorbed by the midgut epithelium of *C. maculatus* larvae (Uchôa et al., 2006; Souza et al., 2010). In the light of these new discoveries concerning the fate of ingested vicilins in *C. maculatus* larvae, we decided to reinvestigate the localization of variant vicilins in the midgut of larval cowpea beetle, taking into consideration the possibility of an intracellular target for the absorbed variant vicilins.

In this work we traced the fate of FITC-labelled vicilins from susceptible and resistant cowpea seeds following ingestion of seed flour by *C. maculatus* larvae. Bioassays confirmed that the IT81D-1053 cultivar is resistant to our strain of *C. maculatus* (Fig. 1). It has been demonstrated that the resistance caused by IT81D-1053 involves antibiosis (Soares, 2012). Experiments confirmed the transport of both forms of vicilins from the midgut lumen into the haemolymph and fat body cells of first and fourth instar larvae (Figs. 2 and 3). In one set of experiments, the larvae were fed for 24 h and whole larvae were prepared for microscopy. There was no difference between the fluorescence found in the midgut tissue or fat body in animals fed with either susceptible or resistant vicilins (Figs. 2 and 3). FITC–vicilin complexes appeared to be concentrated in vesicular structures in the cytoplasm of enterocytes and in fat body cells within 24 h of ingestion (Figs. 2–4).

Determinations of vicilin concentration in the whole bodies and midgut of growing *C. maculatus* larvae showed that since the start of the feeding activity, these animals transport vicilins to their internal environment and vicilins could also be detected in the fat body and gonads of adults (Souza et al., 2010; Alexandre et al., 2011). The quantitative determinations (ELISA), FITC–vicilin complex localization by confocal microscopy and Western blotting analysis confirmed that vicilins pass from the follicle cells to the oocytes and from the male to the female as a seminal nuptial gift (Alexandre et al., 2011). Souza et al. (2010) suggested that vicilins deposited in the eggs may act as protective compounds against pathogens. These results emphasize the adaptive value associated with vicilin absorption in *C. maculatus*. The absorption pathway of these molecules could be "exploited" by leguminous seeds as target for developing resistance, through the modification of vicilin expression and composition. The toxic effect of variant vicilin ingestion is chronic, not acute, as can be seen in Fig. 1. Even after several days feeding on variant vicilin containing seeds, larvae were still alive, although their development was arrested. Fig. 5 shows that variant vicilins are internalized and remain in the enterocytes longer than control vicilins. Under normal conditions, the larvae cannot change their diet and the alteration of vesicle trafficking can affect the midgut physiology, leading to slow development and eventually death of the larvae.

The absorption of intact proteins across the insect midgut epithelium has been the subject of recent attention and was reviewed by Jeffers and Roe in 2008. Until that time, the literature supported the paracellular over the transcellular route as the main pathway involved in the absorption of proteins by insect midgut cells (Jeffers and Roe, 2008). However, recent studies have provided evidence that endocytosis mediated by receptors is also common. There is growing evidence to indicate the role of membrane proteins that function as receptors in the

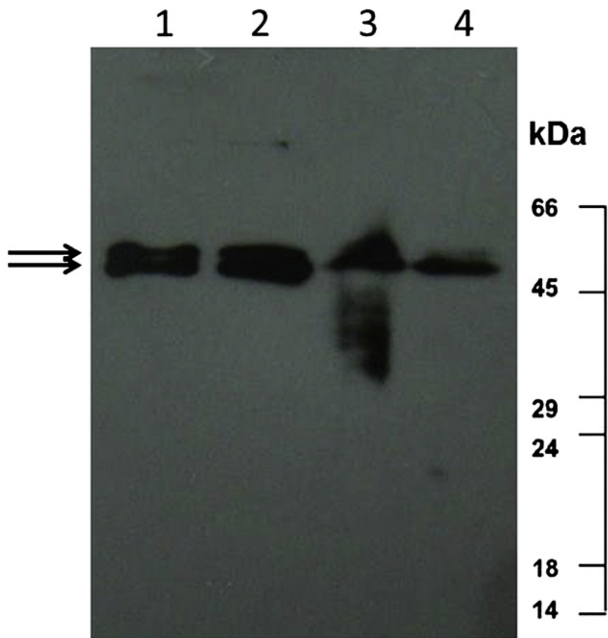

Fig. 6. Western blotting analysis of vicilins from the midgut epithelial homogenates. Larvae fed on Fradinho grains. Lane 1: vicilin standard from the resistant variety IT81D-1053 (0.3 μg protein); lane 2: vicilin standard from the susceptible variety *Fradinho* (0.3 μg protein); lane 3: midgut epithelium soluble fraction (30 μg protein); lane 4: midgut epithelium particulate fraction (30 μg protein). The midgut epithelium homogenate was centrifuged at 15,000 *g* (15 min; 4 °C) and the supernatant and sediment were resuspended in sample buffer and submitted to electrophoresis and Western blotting; Arrows indicate vicilin subunits (between 45 and 60 kDa).

transport of intact proteins across the midgut wall. In larval *Bombyx mori*, the selective transport of mulberry leaf urease correlates with its ability to bind to brush border membrane vesicle preparations (Sugimura et al., 2001; Kurahashi et al., 2005). Casartelli et al. (2008) demonstrated that the internalization of FITC-albumin by columnar midgut cells of *B. mori* in culture was also receptor-mediated. Furthermore, the putative receptor involved was similar to megalin, a multiligand endocytic receptor, that mediates the uptake of different macromolecules in many types of mammalian epithelial cells (Verroust and Christensen, 2002). The authors also showed that FITC-albumin absorption was clathrin mediated and that the integrity of the cytoskeleton was essential for the endocytic machinery. The absorption of two kinds of lectins from *Sambucus nigra* (SNA) by insect midgut CF-203 cells is also mediated by receptors (Shahidi-Noghabi et al., 2011). Internalization of the FITC-labelled lectins was affected by inhibitors of clathrin- and caveolae-mediated endocytosis, suggesting the involvement of both endocytic routes. Different to the absorption of FITC-albumin by *B. mori* midgut cells, the absorption of *S. nigra* lectin is independent on the cytoskeleton.

Results reported in this study suggest that the absorption of vicilins in enterocytes of *C. maculatus* larvae also involves recognition on the cell surface and vesicular trafficking in the cytoplasm (Figs. 4, 7 and 8). In this study a new polyclonal antibody was used in vicilin immunolocalization in the intestinal epithelium. The antibody recognized the vicilin subunit in samples of both particulate (indicating binding to membranes) and soluble fractions of the intestinal epithelium (Fig. 6). Interestingly the recognition of peptides by the antibody in the soluble fraction suggests that there is proteolysis of vicilins within the enterocytes. It is possible that the interaction of the variant vicilins with the microvillar membrane proteins interferes with the physiology of enterocytes in a similar way to that observed in *Spodoptera littoralis* larvae, which had impaired nutrient absorption due to the binding of a lectin to the surface of the microvilli (Caccia et al., 2012). As vicilin molecules are heterogeneous due to the fact that their subunits are encoded by multigene families and post-translational modifications can occur, some forms of vicilins may have higher affinity for their receptors. It is possible that this binding power, associated to a proteolysis resistance (Sales et al., 2001) may interfere with the dissociation of the complexes in endosome vesicles. Further investigations of the presence of vicilin-binding molecule(s) in the midgut tissue and the precise

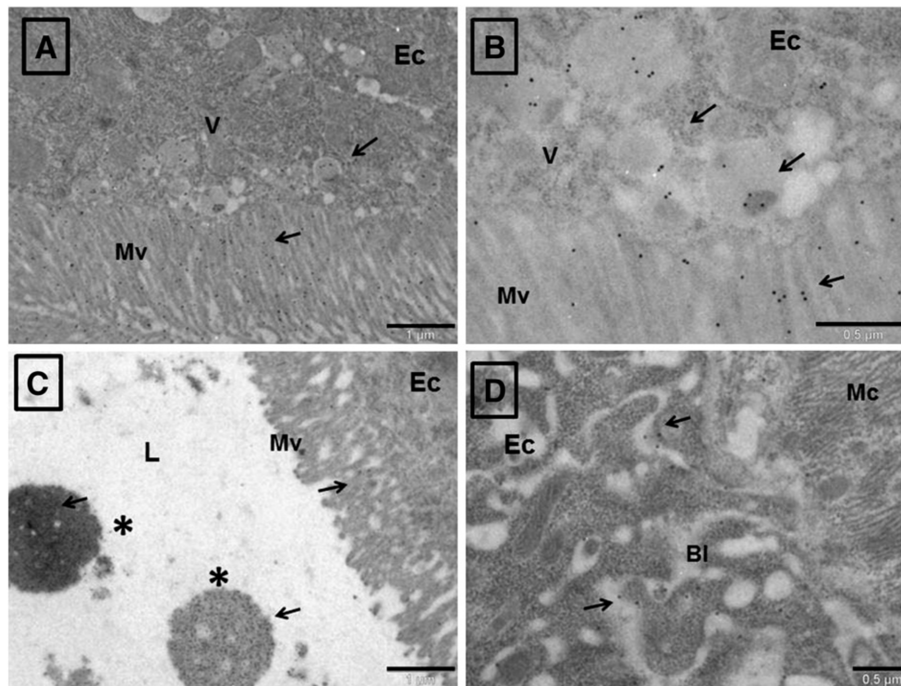

Fig. 7. Immuno-electron detection of vicilins in midgut tissue sections of *Callosobruchus maculatus* larvae fed on susceptible cowpea seeds and immunolabelled with anti-vicilin IgG. Bl: basal labyrinth; L: midgut lumen; Mc: muscle cells; Mv: microvilli; Ec: gut epithelial cells; V: membrane vesicles. A and B: cell apex. Note labelling (arrows) in the microvilli and in membrane vesicles. C: cell apex and midgut lumen interface. Note labelling (arrows) in the microvilli, in membrane vesicles and in circular structures in the midgut lumen (asterisks). D: cell basal portion. Note labelling close to the basal labyrinth (arrows).

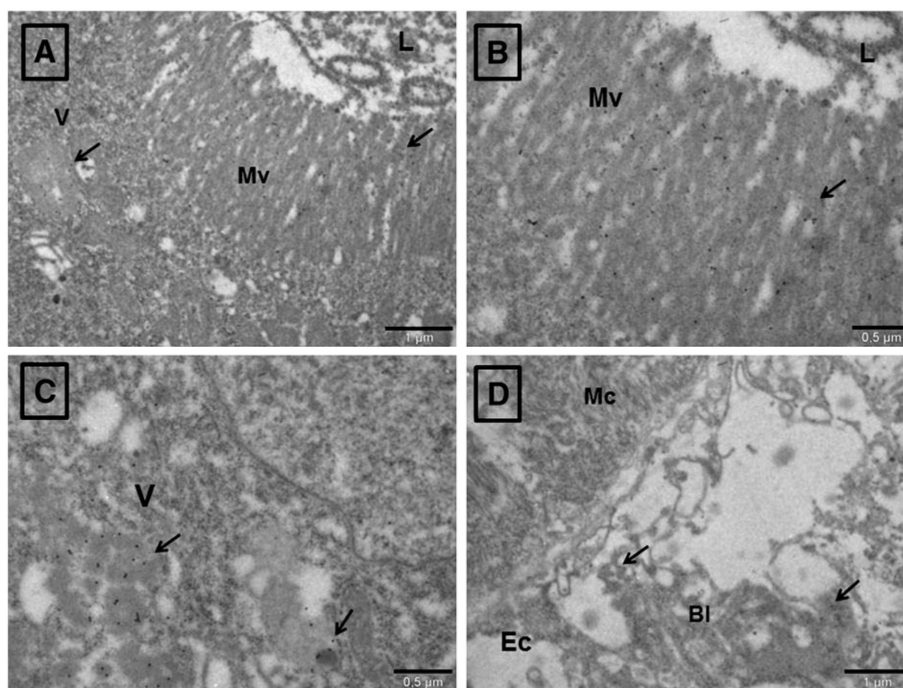

Fig. 8. Immuno-electron detection of vicilins in midgut tissue sections of *Callosobruchus maculatus* larvae fed on resistant cowpea seeds and immunolabelled with anti-vicilin IgG. Bl: basal labyrinth; L: midgut lumen; Mc: muscle cells; Mv: microvilli; Ec: gut epithelial cells; V: membrane vesicles. A and B: cell apex. Note labelling (arrows) in the microvilli and membrane vesicles. C: cell apex and midgut lumen interface. Note labelling (arrows) in the microvilli, membrane vesicles and circular structures in the midgut lumen (asterisks). D: cell basal portion. Note labelling close to the basal labyrinth (arrows).

endocytic route involved are already under investigation in our laboratories and this will hopefully increase our comprehension of the mechanism of action of cowpea variant vicilins and other 7S globulins that bean beetles are not adapted.

Acknowledgements

We thank Chirle Ferreira, Demétrio G. Alves, Emily D. dos Santos and Waldir Caldeira for expert technical assistance. This work was supported by the Brazilian research agencies CNPq, CAPES, FAPESC and FAPERJ. R.B. Leal, R.I. Samuels, M.L.R. Macedo, A.F. Ribeiro, T.B. Grangeiro, C.R. Carlini, W.R. Terra and C.P. Silva are research fellows of CNPq.

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