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Molecular Characterization of Mutations in *Anticarsia gemmatalis* Cadherin Gene and their Relation to *Bacillus thurigiensis* Resistance

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ABSTRACT

Anticarsia gemmatalis Hübner, 1818 is the main soybean defoliating pest in Brazil. The biological control of the species is done with products based on toxins produced by *Bacillus thurigiensis* (*Bt*), as bioinsecticides, or in transgenic plants. After activation by intestinal proteases, these toxins interact with receptors, especially cadherin, leading to death due to the formation of cellular pores. In recent years resistant populations have been identified in the laboratory, which can be a problem if the same patterns are found in crops, reducing their control effect. In this paper, we performed a comparative structural analysis of a mutation region for the gene of this receptor in *A. gemmatalis*, among resistant and susceptible strains treated with a toxin produced by *Bt* (Cry1Ac). The HaCad fragment of the cadherin gene was amplified by PCR, sequenced, and analyzed by bioinformatics tools. The PCR results were positive for resistant specimens but not for susceptible strains, suggesting the presence of a mutation in the resistant strain. In the sequenced fragments of the resistant insects, six haplotypes were found, and the originated amino acid sequences demonstrated the modification in four sites, which did not interfere with the three-dimensional shape of the protein. These data showed considerable variation taking into account the size of the fragment, even if they do not affect the final structure of the protein. The results allowed a better understanding of the mechanisms of resistance to Cry1Ac in the species, mainly in the involvement of cadherin in this process.

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1. Introduction

The soybean crop (*Glycine max* [L.] Merrill) corresponds to the largest agricultural production in Brazil, cultivated in 36.85 million ha [1]. Therefore, this vast area could provide favorable conditions (food, shelter) for pest development and the selection of resistant phenotypes. Among these pests, the velvet bean caterpillar, *Anticarsia gemmatalis* Hübner, 1818, is considered one of the key defoliators together with *Chrysodeixis includens* (Walker 1858) [2]. *Bacillus thurigiensis* Berliner (*Bt*) is a microorganism widely used in the biological control of these species [3]. However, in the past few years, it has been possible to observe a decrease in the effectiveness of *Bt*-based insecticides due to the evolution of resistance in defoliating species [4-6].

Toxins extracted from *Bt* (Cry proteins) interact with membrane receptors, including the cadherin proteins, and can induce the insect's death through an infectious process [7]. In this context, cadherin appears as a key element in the intoxication process, and changes in its structure, as well as in its transcription, translation, and cell location, may result in the non-activation of the insecticide's activity pathway and, therefore, prevent the insect from death, rendering the substance ineffective [8].

The analysis of the possible structural genetic difference focused on candidate genes responsible for resistance between resistant and susceptible individuals is essential since this information may allow future strategies that allow an adaptation in the control of this species. In this paper, we performed a structural analysis of a mutation in the cadherin gene for *A. gemmatalis*, to understand if this mutation may be one of the factors involved in the process of resistance to Cry1Ac.

2. Material and Mmethods

2.1. Insect Collection, Maintenance and Bioassays

The insects used in this paper were the same samples studied in work performed by Pezenti *et al*. [21]. Field populations of velvet bean caterpillar (900 fourth to fifth instar larvae) were collected from non-*Bt* soybean in Sertanópolis-PR, Brazil, in 2011. The larvae were brought into the laboratory and maintained until the pupal stage on soybean leaves. Eighty pupae (40♂+40♀) were placed in acrylic cages, lined internally with sulfite paper as a substrate for oviposition. Adults were fed a 10% honey, 6% sucrose, 0.1% ascorbic acid, and 0.1% methylparaben solution in distilled water. Until hatching, eggs were collected daily and kept in environmental chambers at 26°C. Neonates larvae were reared at 26 ± 1.5 °C, 70% ± 10% r.h. with 14:10 (light:dark) cycle on an artificial diet. In the F199 generation, the larvae were divided into two subpopulations; one was selected at every generation, with the bacterium *Bt* HD73 (pressure of 60%-80% mortality), and another left unchallenged (control). The diet with HD73 was available to the insects for 48 hours, and later the surviving caterpillars were transferred to a diet without the presence of the bacteria to complete their life cycle [9]. In parallel, the susceptible population was maintained in the same conditions and used as a reference to establish the resistance ratios.

Three groups (three replicas) of 16 neonate larvae challenged with seven concentrations, at approximately every 10 generations, were bioassayed to check resistant ratios by incorporating lyophilized *Bt* HD73 into the diet without formalin and antibiotic after it cooled to 50 °C. The assays were performed in triplicate using the following concentrations 140; 76; 41; 22; 12; 6,5 and 3,5 µg/ml of diet for the susceptible population and 480; 260; 140; 76; 41; 22; and 12 µg/ml of diet for the resistant population; in both cases an untreated group established the control.

The larvae were observed daily until the seventh day post-bacterial exposure. Log-dose–probit regression lines and associated parameters were calculated with the Polo Plus probit analysis program LeOra software (POLO, LeOra Software, Berkeley, USA). Resistant ratios at this opportunity were 192-fold higher (LC50 resistant population/LC50 susceptible population) than that observed in the susceptible populations.

For this study, 130 specimens of *A. gemmatalis* (80 resistant and 50 susceptible to the toxin Cry1Ac) were used. The caterpillars were euthanized and stored in an ethanol-ether solution in microcentrifuge tubes at −20ºC. For both groups, the following techniques were performed in a similar way.

2.2. DNA Extraction and Amplification

DNA extraction was performed according to Suzuki *et al*. [10]. The extraction was checked using a 1.5% agarose gel, and the DNA obtained was quantified using NanoDrop®. The amplification of the cadherin fragment followed that proposed by Xiao *et al*. [11] with the HaCad forward primers 5'-GGA GGC AAT TCG GGT GAAC-3' and HaCad reverse primers 5'-ACA TTA ACA GTG ACA GTG AGA GTAG-3' [11]. For the PCR reaction, 0.5µl of DNA from the sample were used at 100ng/µl, 1µl of each primer, 1µl of dNTP Mix (dATP; dCTP; dGTP; dTTP and H_2O Milli-Q) at 10 mM/µl, 0, 5µl of TaqPolymerase Platinum enzyme, 2.5µl of $MgCl_2$ 10x Buffer, 0.8µl of 10mM $MgCl_2$ and H_2O Milli-Q to complete the reaction to 25µl. The samples were subjected to temperature cycles in the thermocycler, following: 1 cycle of 1 minute at 95ºC, 32 cycles (1.4 minutes at 95ºC, 40 seconds at 59ºC for annealing the primers and 1 minute at 72ºC for elongation), and 1 cycle of 5 minutes at 72ºC. After the reaction, the samples were checked in a 1.5% agarose gel and quantified in a NanoDrop®. To ensure the fragments' quality, the samples were submitted to purification with 7.5M ammonium acetate and absolute ethanol, followed by centrifugation, and then washed with 70% ethanol. Then the fragments were eluted in H_2O Milli-Q.

A PCR was performed using primers from the mitochondrial gene ND4 (primers ND4F: 5'-ATT GCC TAA GGC TCA TGT AG-3' and ND4R: 5'-TCG GCT TAG TAG TAG ATTC-3') to ensure that the susceptible result was not the result of errors during the amplification process [12]. For the PCR reactions, 1µl of DNA from the sample were used at 100ng/µl, 1µl of each primer, 1µl of dNTP Mix (dATP; dCTP; dGTP; dTTP and H_2O Milli-Q) at 10 mM/µl, 0,5µl of TaqPolymerase Platinum enzyme, 2.5µl of 10× Buffer, 1,5µl of 50mM $MgCl_2$ and H2O Milli-Q to complete the reaction to 25µl. The samples were subjected to temperature cycles in the thermocycler, following: 1 cycle of 2 minutes at 94ºC, 35 cycles (1 minute at 94ºC, 30 seconds at 56ºC for annealing the primers, and 1 minute at 72ºC for elongation), and 1 cycle of 7 minutes at 72ºC.

2.3. Sequencing and Analysis

PCR products were sequenced automatically (ABI 3500 XL Applied Biosystems). Clearance of DNA sequences, sequence quality analysis, and assembling was performed in the Mega 7.0 software [13] and BioEdit v.7.2.6.1 [14]. The consensus sequences were compared to other sequences previously deposited in the National Biotechnology Information Center (NCBI) Database using the BLAST Search tool. The sequences were aligned in the BioEdit v.7.2.6.1, using the ClustalW tool [15], and a haplotype network was created with Network [16]. The three-dimensional protein structure prediction was made through the Translate tool of the ExPASy portal of the Swiss Institute of Bioinformatics (SIB), and the 3D structure was generated from the automatic modeling server SWISS-MODEL [17].

3. Results

Primers for cadherin mutation amplified a fragment of approximately 100bp. The amplification occurred only in resistant specimens (Fig. **1a**), with no reaction result for susceptible ones (Fig. **1b**). The positive result for amplifying the ND4 mitochondrial gene proved the non-annealing of the study primers for susceptible specimens (Fig. **1c**).

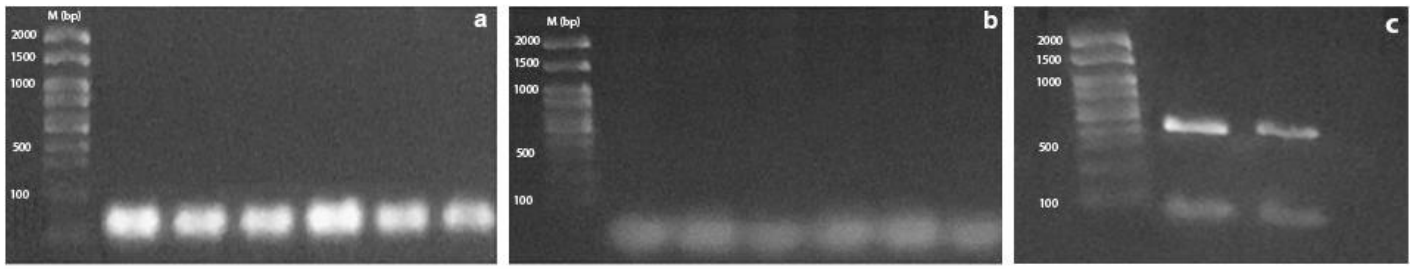

Figure 1: Analysis of amplification of HaCad fragment of *Anticarsia gemmatalis*. (a) Agarose gel (1.5%) for checking the amplification in resistant and in susceptible (b) for the Cry1Ac toxin; and (c) for ND4 mitochondrial gene. The numerical markings (M) indicate the size in base pairs (bp).

Sequencing showed a product with 95 bp (Fig. **2**), and the structural comparison of the sequences showed a variation in 11 positions (Fig. **3a**). These data allowed the formation of six haplotypes (H1 - H6). These haplotypes were plotted on a tree (Fig. **3b**), suggesting a high variation in the sequences, with three transitions (C↔T [11, 95] and T↔C [72]) and eight transversions (A↔C [19], T↔G [31, 60], G↔T[36], C↔G [49], A↔T[53, 68], G ↔C [86]).

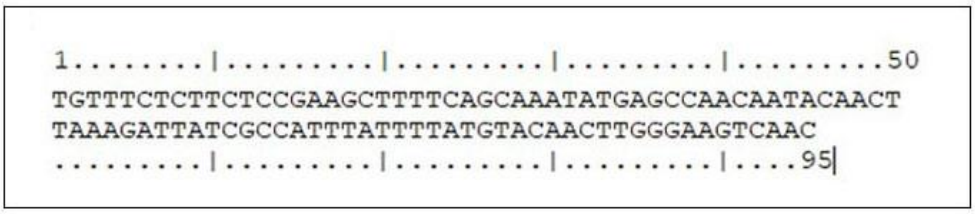

Figure 2: Sequence of the HaCad amplified fragment from *A. gemmatalis*.

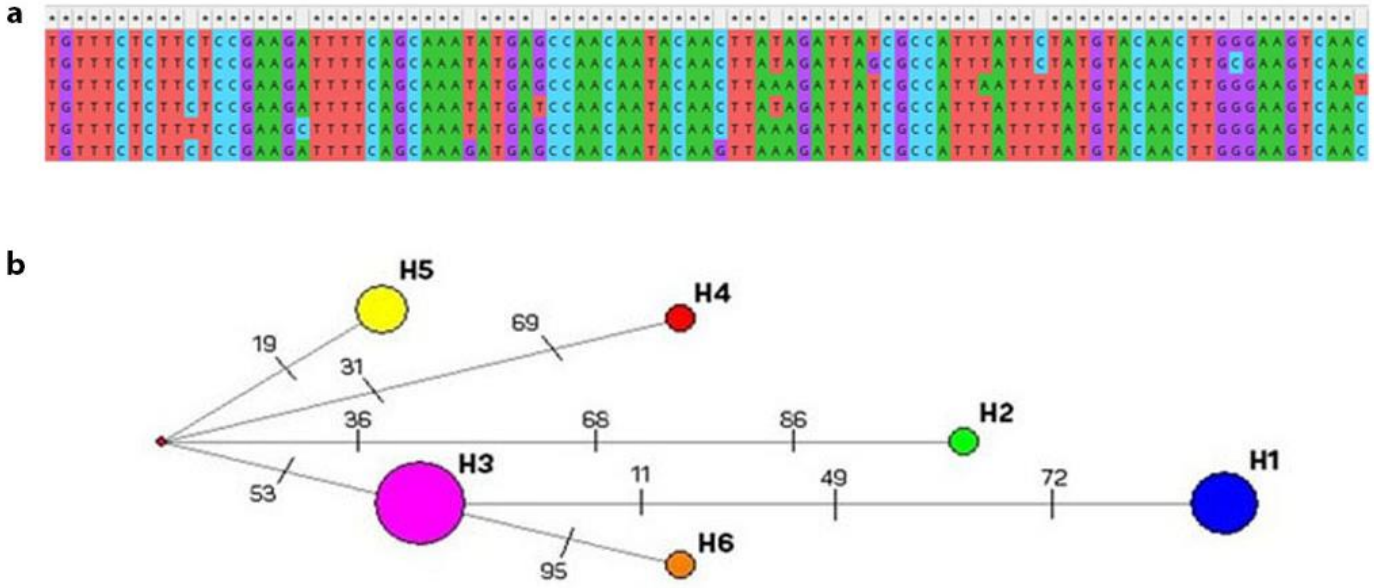

Figure 3: (a) Alignment of the amplified fragments of the HaCad gene from *A. gemmatalis* showing the variation at 11 positions. (b) Haplotypes network obtained for the HaCad fragment. The haplotypes are indicated by H1-H6 and the markings on the lines indicate the position of the nucleotide change.

Regarding the prediction of protein structure, it was possible to observe four peptide sequences. Among the four sequences, only one amino acid changed at different positions in each peptide (Fig. **4a**). Despite this, this structure may not represent the actual structure of the peptide chain. Since it deals with a specific region, anterior or posterior portions not considered may imply the codon reading process and thus change the amino acid sequence. The data found show that even with structural genetic changes and the amino acids formed, the predicted structure of the protein did not change (Fig. **4b**), the protein tends to be morphologically structured for the species, and the genetic changes did not affect the final product.

4. Discussion

Cadherin appears as one of the central points in understanding insensitivity to toxins, especially to Cry1Ac [18]. Our results showed that the amplified fragment in resistant samples represents a part of the Cry1Ac toxin binding site region. The non-amplification of this fragment in susceptible specimens demonstrates that this region is structurally altered between the two groups. The amplification conditions were the same in both groups, and both showed a positive result when submitted to an amplification reaction with mitochondrial ND4 gene primers. Confirming that the susceptible specimens do not present the mutation for the *locus,* this result demonstrates that there were no errors in the amplification.

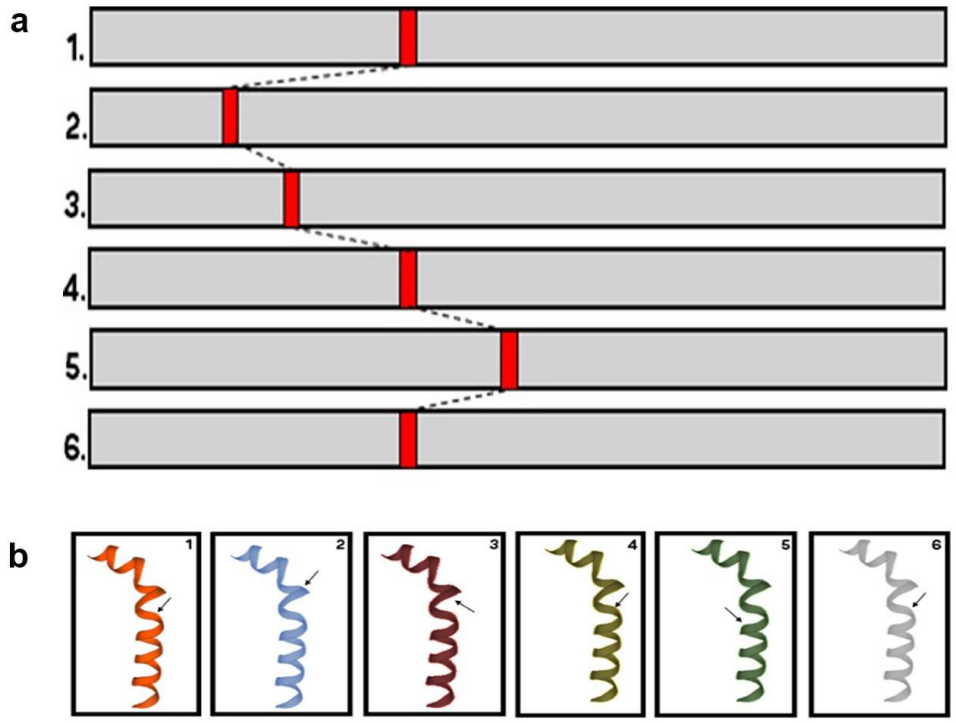

Figure 4: (a) Representation of the distribution and location of amino acid changes in the peptide chain of the HaCad fragment. The red rectangles represent the amino acid change site. Note that sequences 1, 4 and 6 show the same pattern. (b) Three-dimensional representation of the protein prediction for the fragment in *A. gemmatalis*. The numbers indicate the peptide sequence. Note that the structures remain the same for all amino acid sequences. The arrows indicate the variable region of the peptide sequences.

Genes can influence the resistance process in several ways, such as by altering the genetic structure (as may have occurred in this study), transcription, and translation [19] or post-translational alterations [11, 20]. The primers used here allowed us to analyze possible changes in this fragment, and with the results obtained, it can be inferred that cadherin plays an important role in this process for the species, and structural differentiation may be linked to resistance to Cry1Ac.

Transcriptional analyses carried out by our research group revealed no significant differences in the cadherin expression pattern between resistant and susceptible specimens of *A. gemmatalis* [21]. This demonstrates that even though the fragment is mutated, as suggested here, it continues to be transcribed and occupy its basal cellular protein anchoring and adhesion function [22]. However, transcriptional differences between other genes are involved in the resistance process [21]. Thus, it can be inferred that a set of changes lead to insensitivity in resistant individuals and that the mutation in cadherin is an important, but not the only, element involved in this process.

The haplotype network demonstrated that although there is a high similarity in the sequence, resistant individuals tend to diverge at specific points, and this tendency is greater for crossings than transitions. In addition, the number of haplotypes found is relatively high, considering the length of the sequence. The amplified fragment showed a well-maintained pattern for the resistant strain, demonstrating a high fixation of the mutation in this group. However, the occurrence of many transversions and transitions within a short fragment demonstrates that the structure of the gene tends to have a considerable number of nucleotide variations.

The predicted structure of the protein did not change, and the genetic variation did not affect the final product. However, as with the peptide sequence data, since the interaction of the protein fragment formed with other regions of the total protein can be changed, it is important to note that the absence of the anterior and posterior regions can alter the amino acid sequence as well as the three-dimensional protein formation.

Zhang *et al*. [8] reported a deletion in the extracellular portion of cadherin in *Helicoverpa armigera*, in field and laboratory populations, corroborating the idea of a stable structure of the mutated fragment. The preservation of the protein structure is an important fact in the fixation of the mutation [23].

Despite their potential, indiscriminate use, and the lack of species-specific control, bio-insecticides can end up becoming unfeasible as new resistant populations emerge [24]. The structural differentiation of cadherin and the transcriptional differences between resistant and susceptible individuals appear as good indicators to improve monitoring population control in crops and how the insecticide cytotoxic effect is characterized. While the resistance is a complex process and the consequence of biological adaptations and human actions, our results corroborate the idea that the cadherin gene may be implicated in the non-susceptibility of Cry1Ac-resistant specimens.

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