MOLECULAR CLONING OF A NEW CRY8 GENE OF A *Bacillus thuringiensis* STRAIN HIGHLY EFFECTIVE AGAINST COTTON BOLL WEEVIL (*Anthonomus grandis*)


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RESUMO

Cotton boll weevil (*Anthonomus grandis*) is considered the key pest of cotton in many countries in North and South America. Insecticides have been largely used, but control remains difficult because of the larvae endophytic behavior. The use of *Bacillus thuringiensis* (Bt) endotoxins arises as an efficient alternative of control. From a Bt strain bank from EMBRAPA-CENARGEN, we characterized a new strain with strong activity against boll weevil larvae. With the aim to identify the Cry proteins active against this pest, biochemical characterization was performed showing the presence of proteins in the 60 kDa and 20-30 kDa range, while electronic microscopy showed bipyramidal and spherical crystals. Based on specific primers for cry8 genes combined with TAIL-PCR technique, a new Cry8 gene was isolated. In total 2688 bp were amplified comprising 896 amino acids. The N-terminal and C-terminal extensions of the new Cry8 endotoxin are highly similar to other Cry8 proteins, while the three structural domains are less conserved, particularly the second and third domains, which are involved in receptor binding, suggesting novel insecticidal activities/specificities for the isolated gene. Current work is now being directed towards the expression of the new Cry8 gene to access its effect against cotton boll weevil.

INTRODUCTION

Cotton boll weevil (*Anthonomus grandis*) is considered the key pest of cotton, causing severe damage in cotton crop in many countries in North and South America. Insecticides have been used as the major form of defense, but control of this pest remains difficult because of the larvae endophytic behavior. The use of *Bacillus thuringiensis* (Bt) endotoxins to control insect pests arises as an efficient alternative of control. The crystal produced by Bt, composed by δ-endotoxin or crystal protein (Cry), presents specific toxic action to the larvae of the insects and are described as being harmless to humans. From a BT strain bank of EMBRAPA-CENARGEN, we characterized a new Bt strain highly toxic to insects in three orders (Diptera, Coleoptera and Lepidoptera), with strong activity against boll weevil larvae. With the aim to isolate novel Cry proteins active against insect pests we have performed a biochemical characterized of this strain and isolated a gene encoding a new Cry8 toxin.

MATERIAL AND METHODS

Biochemical characterization was performed by SDS-PAGE, two-dimensional gel electrophoresis in the 3.5-10 range and scanning electronic microscopy of purified crystal. Crystals were purified by a sucrose gradient. Initial PCR amplifications were performed with total DNA and primers specific for the Cry8 endotoxin family. Further amplifications were performed by TAIL-PCR (Thermal Asymmetric Interlaced PCR) using specific primers based on previous amplified sequence and arbitrary primers. Selected amplified fragments were cloned into the plasmid vector pGEM-T Easy and recombinant clones were sequenced in both strands in an automated DNA sequencer. Computer analysis of the DNA and amino acid sequences were performed using the GCG package (Genetics Computer Group, Inc.), bioinformatics resources of the NCBI homepage (http://www.ncbi.nlm.nih.gov)
and the EBI website (http://www.ebi.ac.uk/). Sequence alignments were obtained using CLUSTALW. Protein modelling was carried out using MODELLER-6.

RESULTS AND DISCUSSION

Biochemical characterization of isolated crystals showed the presence of major proteins in the 60 kDa and 20-30 kDa range, while electronic microscopy showed the presence of bipyramidal and spherical crystals. Based on specific primers for cry genes combined with TAIL-PCR technique, a new gene was isolated. In total 2688 bp were amplified comprising 896 amino acids. The new Bt gene closely aligned with the Cry8 endotoxin family (Fig. 1). The amplified segment comprises the three structural domains characteristics of the δ-endotoxins activated N-terminal, plus about 240 amino acids of the C-terminal extension. Comparisons with the other Cry8 endotoxins indicates that about 260 are still missing from the C-terminal of the new Cry8 protein (Fig. 2).

The N-terminal and C-terminal extensions of the new Cry8 endotoxin are highly similar to those of other Cry8 proteins, while the three structural domains are less conserved, particularly the second and third domains (Table 1), which are involved in receptor binding, suggesting novel insecticidal activities/specificities for the isolated gene. Despite extensive differences in the primary sequence of the three structural domains, a molecular model of the new Cry8 toxin indicates it is similar to the other Cry8 endotoxins. The main difference is the length of two loops joining the apical β-strands in domain II, that may be implicated in receptor binding and account for the specificity of the new toxin.

PERSPECTIVES

Current work is now being directed towards the expression of the new Cry8 gene to access its effect against cotton boll weevil. The availability of the recombinant protein will also allow the isolation and identification of its receptor in the insect midgut. Additionally, novel activities/specificities based on the sequence of the new Cry8 gene can be explored by the use of techniques such as DNA shuffling or point mutations.

Figura 1. Dendrogram of the alignment of the new Cry8 toxin with other members of the Cry8 and Cry9 families. Sequence alignment was performed using ClustalW.
**Figura 2.** Schematic representation of the protein domain structure of the new Cry8 endotoxin and comparison with Cry8Aa.

**Table 1.** Mean identity between the protein domains of the new Cry8 protein with the corresponding domains in other members of the Cry8 family.

<table>
<thead>
<tr>
<th>Cry8 new</th>
<th>N-terminal</th>
<th>Domain I</th>
<th>Domain II</th>
<th>Domain III</th>
<th>C-terminal</th>
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<tr>
<td>Cry8Aa</td>
<td>87.7%</td>
<td>48.6%</td>
<td>29.8%</td>
<td>35.5%</td>
<td>90.7%</td>
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<tr>
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<td>47.7%</td>
<td>31.3%</td>
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<td>91.1%</td>
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<tr>
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<td>57.7%</td>
<td>31.6%</td>
<td>33.3%</td>
<td>68.6%</td>
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