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Production and Characterization of a Subtractive cDNA Library and Quantitative PCR Analysis of *Choristoneura fumiferana* Genes Differentially Expressed in Response to *Bacillus thuringiensis* Cry1Ab Toxin Exposure

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Bacillus thuringiensis is a biological control agent for *Choristoneura fumiferana*, exerting its lethal effect primarily through the production of crystal proteins. There is concern about the impact of Cry toxins on non-target species, especially in terms of sublethal effects. By understanding the transcriptional response of *C. fumiferana* larvae to a sublethal dose of Cry1Ab toxin, we can proceed to assess whether genes showing altered transcriptional profiles can be used as universal Cry toxin stress markers for non-target insects. To this end, a suppression subtraction hybridization library was created and differential mRNA expression of selected clones was measured using a quantitative polymerase chain reaction (Q-PCR) technique.

Spruce budworm (*Choristoneura fumiferana*) larvae are destructive defoliators of North American forests where epidemic episodes result in major damage to spruce and balsam fir trees (1, 2). These episodes have been controlled using formulations containing the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) that contains crystal proteins (Cry) as the active toxic agent. Bt has been developed commercially for the control of various agronomical insect pests (3, 4) and represents an important alternative to chemical insecticides (5).

Although Cry toxins have a relatively narrow host range, their effect on non-target organisms remains a controversial environmental issue (6). Sublethal effects on non-target insects are not readily apparent but can be assessed at a molecular level. By understanding the molecular response of the larvae to sublethal doses of a Cry toxin, we can then proceed to assess whether genes showing altered transcriptional profiles can be used as universal Cry toxin stress markers for non-target insects. The present study describes the construction and partial characterization (sequencing and mRNA quantification) of a suppression subtraction hybridization library between a Cry1Ab toxin exposed larval population of *C. fumiferana* and a control population not exposed to the toxin.

C. fumiferana larvae were divided into two populations: a Cry1Ab protoxin fed population (35 ng of Cry1Ab protoxin) and a control population. Midguts from diet fed instar 4 (L4) larvae were removed by dissection 24 h after the end of the feeding period and immediately stored at -80°C. Treatment was followed by mRNA extraction in order to create a subtracted library using both larval populations (control and toxin Cry1Ab treated). Gene expression was evaluated by Q-PCR analysis of 17 selected unique genes. All Q-PCRs were done in triplicate for two different biological replicates.

The transformed library was initially characterized by sequencing 1091 clones. Among these clones, 623 possessed unique sequences with the remainder representing duplicate or contiguous sequences. BLASTX analyses were performed and 171 clones were found to match to a specific function (e-value less than e^{-15}). Those sequences ascribed a putative molecular function using the Gene Ontology Consortium software (7) and were classified in different categories. The majority of the unique sequences (54%) had a molecular function related to catalytic activity. The two other major functions represented were proteins involved in binding (15%) and structural (15%) functions. A small amount (3%) had a role in the stress response of the insects.

All gene expression experiments (Q-PCR) were normalized to a housekeeping gene, the acidic calcium-independent phospholipase A2 (PLPA2). After Q-PCR analysis, 17 clones were classified in three different groups according to the type of expression observed

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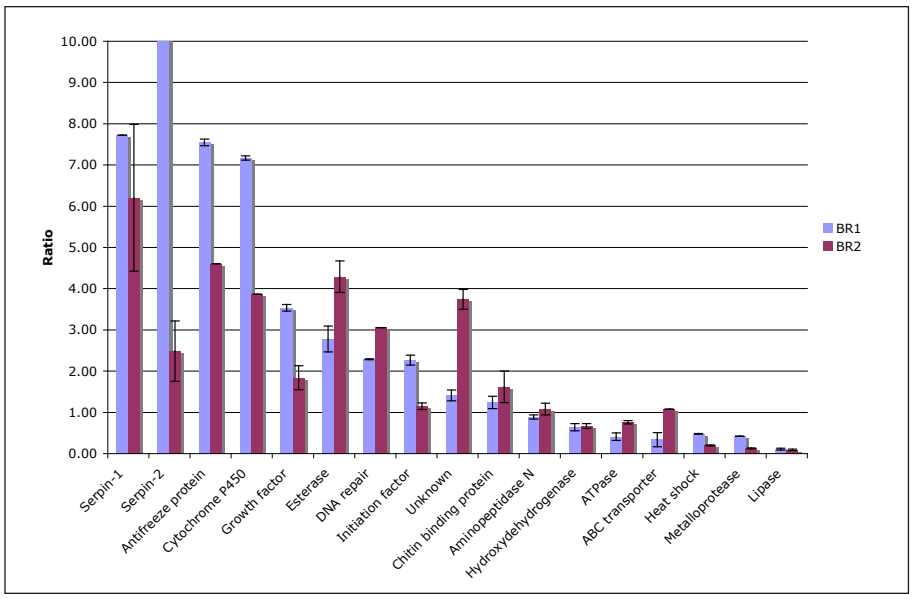

FIG. 1. Levels of mRNA expression by Q-PCR. BR1 and BR2 represent two independent biological replicates of the feeding experiments. Error bars represent standard error. All data were normalized to PLPA2 and expressed as a ratio (toxin fed/control).

after toxin exposure. Nine clones were considered overexpressed (ratio > 1.5), five clones showed stable levels of mRNA and three clones were transcriptionally repressed compared to the control when exposed to the toxin (Fig. 1). Two serine protease inhibitors (serpins) showed an overexpression profile in both biological replicates. Four other clones showed an overexpression profile after toxin exposure: an antifreeze protein, a cytochrome P450, an esterase and a protein involved in DNA repair. Three clones having homology with a growth factor, an initiation factor and a gene of unknown function also showed an enhanced expression profile in one replicate, but their expression levels remained unchanged in the other. This result was presumably due to the fact that only a single time point was examined in both replicates. In other words, the second replicate may have been sampled when the genes were either at the start or at the finish of their altered transcriptional profile. Five clones related to binding activity showed a stable expression profile (ratio between 0.5 and 1.5): an aminopeptidase, a chitin receptor, a hydroxydehydrogenase, an ATPase and an ATP binding cassette (ABC). Clones having homology with a lipase, a metalloprotease precursor and a heat shock protein were all repressed by the toxin treatment (ratio < 0.5).

In this study, it was shown that the response elicited by sublethal Cry1Ab toxin ingestion seems to be related primarily to alterations in metabolic activities in the insect. A large number of enzyme-related genes were either enhanced or repressed while specific Cry toxin receptor genes such as aminopeptidase genes did not seem to have an altered transcriptional profile. Extending the analysis of these expression profiles among other toxin-exposed insects is needed to assess whether this stress response to Cry toxin intoxication is universal.

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