

## Gene Expression Response of the Spruce Budworm, *Choristoneura fumiferana*, after Exposure to Various Doses of *Bacillus thuringiensis* Cry1Ab Toxin Using Microarray Technology

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In order to gain more insight into the response of the North American lepidopteran forest pest *Choristoneura fumiferana* to the Cry1Ab toxin of *Bacillus thuringiensis*, linearly amplified mRNA was synthesized from midguts of healthy and infected insects exposed to various sublethal doses of this toxin. Fluorescently labeled cDNAs obtained from the mRNA were hybridized onto a custom DNA microarray chip containing 1091 clones isolated from a subtractive library between toxin-fed and control insects. Our results show that genes implicated in various metabolic functions were specifically enhanced or repressed when susceptible larvae were exposed to sublethal doses of toxin.

Spruce budworm larvae (*Choristoneura fumiferana*) are major defoliators of many spruce species and are responsible for important economic losses in North America forests (5). Chemical control of *C. fumiferana* is problematic for many reasons (pollution, toxicity to non-target insects, etc.) and, as an alternative, commercial formulations containing Cry toxins of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) have been developed and used for many years as a biocontrol agent in Canadian forests (6). Although it is known that Bt toxins have a narrow and specific host range (2), potential subtle effects on non-target insects, such as altered oviposition, reduction in insect size or slower maturation, may not be readily apparent and these effects could influence insect population dynamics over the long term. In order to evaluate the risk for non-target insects potentially exposed to Bt toxins, we determined the transcriptional response of *C. fumiferana* larvae exposed to various sublethal doses of Cry1Ab toxin using microarray technology.

To this end, a cDNA subtractive library enriched in clones differentially expressed in *C. fumiferana* intoxicated larvae (20 ng Cry1Ab toxin/larva) was created from which 1091 clones were selected and analyzed. Individual PCR-amplified fragments (amplicons) from each of the clones were immobilized onto glass slides in triplicate. A set of positive control genes (phospholipase A2, tubulin of *C. fumiferana* and a spike-in gene,

chlorophyll synthetase of *Arabidopsis thaliana*) was also included on each slide to allow normalization of the data. For microarray analyses, messenger RNA was isolated from midguts of either healthy or exposed (5, 10, 20 and 40 ng Cry1Ab toxin /larva) instar 4 (L4) larvae, one day after removal of diet, linearly amplified and labeled using fluorescent cyanine dyes. The labeled amplified DNA was then hybridized onto the microarray chip and subsequent data normalization, statistical analysis and visualization were performed using GeneSpring software (Agilent Technologies Inc., Palo Alto, CA, USA). Negative control experiments using a coleopteran-specific toxin (20 ng Cry3A toxin/larva) were conducted simultaneously to determine gene response specificity to the lepidopteran-specific Cry1Ab. Independent validation of microarray results was done using quantitative PCR (Q-PCR). Specific primers for three selected clones having significant BLASTX homology with an aminopeptidase, a serine protease inhibitor and a lipase were designed and synthesized in order to amplify a 100-150 base-pair PCR product. SYBR Green I dye (Applied Biosystems, Foster City, CA, USA) was used to quantify each of the relative mRNA levels for each gene, as analyzed by the Rotor-Gene quantification software (Corbett Research, Cambridge, UK). A mathematical model was then used to calculate the relative expression ratio between the toxin-fed and control populations (4).

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TABLE 1: Clones with known functions overexpressed (i.e., intensity ratio toxin/control >1.8) and repressed (i.e., intensity ratio toxin/control <0.5) after toxin treatment.

Treatments (ng/larva)					
	BLASTX homology	5 ng	10 ng	20 ng	Ratio
Overexpressed	Serine protease inhibitor	-	+	++	-: 1-1.8 +: 1.8-2.5 ++:> 2.5
	Esterase	+	+	+	
	Cytochrome P450	-	+	++	
	Tumor protein	+	+	+	
Repressed	Hydrolase activity	++	++	++	-: 1-0.5 +: 0.5-0.4 ++:< 0.4
	- Glucosidase	+	+	+	
	- Lipase	-	+	++	
	- Carboxypeptidase	-	-	+	
	- Serine protease	-	-	-	

Microarray results showed that 24 clones were specifically enhanced after a 20 ng toxin/larva treatment. In particular, several clones having sequence homology with the genes of serine protease inhibitors (serpins) as well as that of a cytochrome P450 protein were enhanced after a 10 ng toxin/larva treatment (Table 1). Serpins are involved in the defense immune system of insects as they inhibit serine protease activity (1) and the cytochrome P450 superfamily proteins is known to be involved in a variety of metabolic processes including insecticide metabolism and, consequently, insecticide resistance (3). We were able to detect 124 clones specifically repressed after a 20 ng toxin/larva treatment (Table 1). Moreover, 103 clones were already specifically repressed after a 5 ng toxin/larva treatment. For the most part, repressed clones shared sequence homology with the genes of proteins implicated in hydrolase activity (lipase, glucosidase, carboxypeptidase, midgut serine proteases) (Table 1). Q-PCR analysis on the selected

lipase, serpin and aminopeptidase genes, showed that indeed the lipase gene was repressed while the gene coding for the serpin was overexpressed after the toxin treatments. In the case of aminopeptidase, a known receptor for Cry1Ab toxin in *Lepidoptera*, its expression was unchanged after the various toxin treatments (Fig. 1).

In conclusion, we show that the parallel processing power of DNA microarray technology is a powerful and useful tool for screening large numbers of genes for altered gene expression. Using a susceptible insect, we show that many genes involved in several metabolic functions have their expression ratio altered by toxin treatment, even at low dose.

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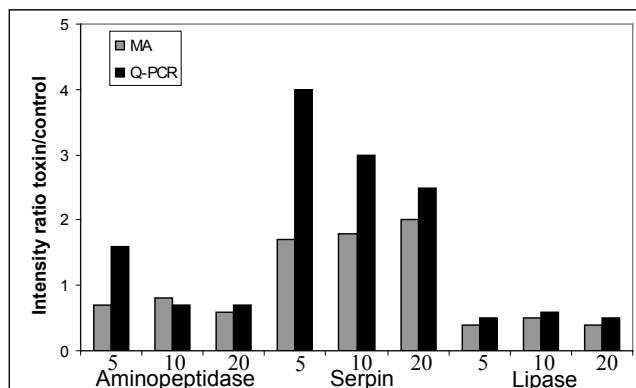


FIG. 1: Comparison of microarray and Q-PCR results. Analysis was done in triplicate for each toxin treatment (5, 10, 20 ng/larva).