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GENETIC CHARACTERIZATION OF A COLORADO POTATO BEETLE STRAIN RESISTANT TO THE COLEOPTERAN SPECIFIC -ENDOTOXIN OF *Bacillus thuringiensis* subspecies *tenebrionis*

presented by

Utami Rahardja

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GENETIC CHARACTERIZATION OF A COLORADO POTATO BEETLE STRAIN RESISTANT TO THE COLEOPTERAN SPECIFIC δ-ENDOTOXIN OF *Bacillus thuringiensis* subspecies *tenebrionis*

by

Utami Rahardja

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

Department of Entomology

1995

ABSTRACT

GENETIC CHARACTERIZATION OF A COLORADO POTATO BEETLE STRAIN RESISTANT TO THE COLEOPTERAN SPECIFIC δ-ENDOTOXIN OF *Bacillus thuringiensis* subspecies *tenebrionis*

By

Utami Rahardja

The inheritance of Colorado potato beetles, *Leptinotarsa decemlineata* (Say), resistance to *Bacillus thuringiensis* CryIIIA δ-endotoxin was investigated. Analysis of probit lines from the F_1 reciprocal crosses indicated that inheritance of *B. thuringiensis* resistance was not influenced by the sex of the parents. The degree of dominance (D) of 0.77 and 0.76 for the (R X S) and (S X R) F_1 generations, respectively, indicates that *B. thuringiensis* CryIIIA δ-endotoxin resistance is conferred by a partially dominant gene(s). χ^2 analysis of mortality responses of backcrossed offspring suggested that resistance might be caused by more than one genes. When the selection pressure was removed from colonies being selected for resistance, the resistance level of the selected colony decreased after five generations. If selective pressures were for another 12 generations, resistance levels did not revert to the resistance levels observed in susceptible colonies.

The realized heritability of resistance to *B. thuringiensis* CryIIIA δ-endotoxin Colorado potato beetles was estimated for over 29 generations. The estimates reached the highest value at generation four, and then decreased and reached values with very little fluctuation after the tenth generation. The heritability estimates in the first 12 generations

showed significant correlation with the increment of the resistance ratio. There was no significant correlation between standard deviation of LC_{50} and the number of generations over 29 generations in both selected and unselected strains. The mean estimated heritability value after 29 generations is relatively low ($h^2 = 0.10$).

DNA markers for genes conferring resistance to *B. thuringiensis* CryIIIA endotoxin in Colorado potato beetles were developed by means of Polymerase Chain Reaction (PCR) technique. Primers R-14 (5'-ACAGGTGCTG-3') and R-17 (5'-CCGTACGTAG-3') gave 650 and 1800 basepairs fragments, respectively, which are specific markers for the resistant in our laboratory colony. The linkage between the genetic markers for the dominant gene(s) and the resistant phenotype was determined. The 650 basepairs marker was not linked to the gene conferring resistance to CryIIIA endotoxin. The 1,800 basepairs fragment identifies a genetic region that significantly linked to the gene. The recombination fractions of the 650 and 1,800 fragments were 0.46 and 0.20, respectively. Population of Colorado potato beetle from nine different locations at Michigan were used to validate the usefulness of the primers (R-14 and R-17) for field detection. The susceptibility of the field samples were determined and a total of 1000 field sampled beetles were tested. Nine field populations in Michigan tested with R-14 showed the diagnostic marker representing 0.02% of the total populations tested. Two of the nine field populations tested with R-17 showed the diagnostic markers. The R-17 marker appears to be reliable in detecting resistant population.

Ten different 10 to 15 oligomer primers used in twenty five different combinations of primers were used to detect polymorphism through Reverse-Transcript-Polymerase

Chain Reaction (RT-PCR) analysis. Two sets of the primers exhibited polymorphism that were related to the resistance to *B. thuringiensis* CryIIIA endotoxin in Colorado potato beetle.

"And I will restore to you the years that the locust hath eaten, the cankerworm, and the caterpillar, and the palmerworm, My great army which I sent among you. And ye shall eat in plenty, and be satisfied, and praise the name of the LORD your God, that hath dealt wondrously with you: and My people shall never be ashamed. And ye shall know that I am in the midst of Israel, and I am the LORD your God, and none else: and My people shall never be ashamed. And it shall come to pass afterward, that I will pour out my Spirit upon all flesh....."

Joel 2: 25-28

To my mother and father, Sumijati and Rahardjamulja

who introduced me to the beautiful and interesting creature,

insects

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My deepest honor goes to God for His faithfulness and provisions. He has given me the opportunity to study insects, the creature that I was interested in and has helped me to fulfill my goal.

"Trust in the Lord with all your heart and lean not on your own understanding."
Proverbs 3:5

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Colorado Potato Beetle: The Need for Better Pest Management

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), was first described by Thomas Say, a young entomologist at the Philadelphia Museum of Science, in 1823. The beetle was found feeding on a weed, *Solanum rostratum*, growing in arid areas of the Colorado River basin in the western United States (Berenbaum 1983). It is suggested that the Colorado potato beetle and its host plant originated in Mexico. The beetle invaded cultivated potatoes (*Solanum tuberosum* L) when settlers moved west and planted potatoes in the foothill of the Rocky Mountains (Casagrande 1985). Approximately 30 years after first being identified, the Colorado potato beetle was considered as a pest of potatoes in the western United States. It was particularly a problem in western part of Missouri. The Colorado potato beetle is now considered the most destructive pest of potato in many parts of the United States.

A variety of approaches have been used to control the Colorado potato beetle. Chemical control was initiated when the first synthetic stomach poison, Paris Green, was invented in the 1860's. This copper acetoarsenate became the leading insecticide for many leaf-feeding pests, including the beetle, until the discovery of lead arsenate. DDT was introduced during World War II, and was adopted as the universal insecticide. This insecticide was very effective for controlling potato beetles and was widely used by potato growers across the country (Gautheir et al. 1981). Following the failure of DDT to control this pest, Dieldrin showed a significant degree of insect control during the 1950's (Hofmaster 1965). During the 1960's, azinphosmetyl and carbaryl were widely used with excellent results for a number of years (Gautheir et al. 1981). Currently, the controls of

the Colorado potato beetle rely heavily on multiple applications of insecticides, cultural control, bacterial insecticides and even flaming with propane devices.

Insects, like any other organism, evolve through selection as a result of their changing environment. A population becomes adapted through natural selection to its environment. Resistance may be an unavoidable consequence of insecticide use. Insect pests have evolved mechanisms that enable them to circumvent toxic agents. The gene pool of most of the insect pests may contain genes that enable the insect pests to detoxify or resist the toxic agents.

Heavy reliance on insecticides for control has resulted in insecticide resistance, environmental contamination, and suppression of natural enemies. Many insect pests have adapted to chemical controls by developing metabolic and behavioral defense mechanisms against insecticides. Insect resistance to insecticides was reported over 70 years ago, but the greatest increase and most important development in resistance has occurred during the last 40 years since the discovery of synthetic insecticides (reviewed by Georghiou 1986). The Colorado potato beetle was one of the first pests to exhibit resistance to DDT (Gautheir et al. 1981). Insecticides often failed to control Colorado potato beetles within one or two years of introduction because of cross-resistance (Forgash 1981, Grafius 1986). To date, in many parts of the United States, the Colorado potato beetle has developed resistance to all classes of conventional insecticides including organochlorines, organophosphates, carbamates, and pyrethroids (Gautheir et al. 1981, Georghiou 1986, Grafius 1986, Ioannidis et al. 1991, 1992).

Bacillus thuringiensis δ-endotoxin: A Promising Bioinsecticide?

Microbial insecticides, such as *B. thuringiensis* δ-endotoxins, have been considered the best alternative for controlling insect pest populations. The δ-endotoxins

have limited specificity (Haider et al. 1986) and have no known detrimental effects on humans or wild or domestic animals and they tend to be short-lived in the environment (Stone et al. 1989, McGaughey & Beeman 1988). Therefore, these bioinsecticides are attractive alternatives to chemical insecticides which are perceived to be more environmentally disruptive and hazardous to applicators and consumers. In addition, the limited range of activity of the δ-endotoxins toward insects means that often they will kill the target pest species but have no effects on predatory species. This feature makes them highly desirable for use as components in integrated pest management (IPM) programs.

A group of bacteria possesses insecticidal activities. This includes species with the ability to infect healthy insects and rapidly multiply in their hosts following the infection. The major species of bacteria with those abilities are spore forming bacilli. Among the spore forming bacilli, *B. thuringiensis* and *B. sphaerius* are two major groups that produce protoxin.

Bacillus thuringiensis is an aerobic spore forming Gram positive soil bacterium. The crystal δ-endotoxin is produced as large protein molecules during sporulation. These proteins are deposited as parasporal inclusion. The crystalline inclusion, also known as δ-endotoxin, is a gut poison for larva of more than 100 insect species belonging to the order of Lepidoptera, Diptera and Coleoptera (Höfte & Whiteley 1989).

Many toxic proteins with varying similarity, or degrees of homology in amino acid sequence, have been isolated from worldwide collections of *B. thuringiensis* strains (Höfte & Whiteley 1989, Ishii & Ohba 1993, Kaelin 1994). These proteins are encoded by the *cry*I, *cry*II, *cry*III, *cry*IV and *cyt* genes. All of these *cry* δ-endotoxin genes are thought to have a common evolutionary origin because of the highly conserved amino acid composition. The C-terminal part of CryI, CryIVA and CryIVB δ-endotoxins that does not contain toxic segments shows the most highly conserved domain of the protein. The N-terminal half of the protein is essential for toxicity. In the amino acid sequence

corresponding to the toxic domain, five highly conserved sequence fragments can be distinguished in all but CryIIA, CryIIB and CryIVD δ-endotoxins. These fragments are separated by highly variable sequences of various lengths for different crystal proteins. With the exception of CryII and CryIVB δ-endotoxins, a stretch of hydrophobic amino acids at a comparable position within the 120 N-terminal amino acids of all δ-endotoxins is remarkably conserved. This feature indicates a significant function. It has been proposed that the conserved hydrophobic region plays a role in an interaction between the δ-endotoxins and the membrane of midgut epithelial cells (Schnepf et al. 1990).

A protoxin comprising 130 - 140 kDa polypeptides is the predominant parasporal component of most *B. thuringiensis* subspecies. Digestion process yields smaller proteinase resistant δ-endotoxins which are derived from the N-terminus of the protoxin (Höfte et al. 1986, Choma & Kaplan 1990). CryI (lepidopteran δ-endotoxins) in general, CryII (lepidopteran and dipteran δ-endotoxins) and CryIV and cytA (dipteran δ-endotoxins) protoxins are solubilized and digested into smaller proteins ranging from 30-80 kDa (Lilley et al. 1980, Huber & Lüthy 1981, Höfte & Whiteley 1989). In contrast, CryII, CryIII and CryIVD δ-endotoxins do not undergo protease mediated C-terminal degradation. Three subspecies of *B. thuringiensis*, known to produce coleopteran-specific δ-endotoxin, release crystaline protein that does not need to be further cleaved to activate the δ-endotoxin (Caroll et al. 1989, Herrnstadt et al. 1986). These proteins appear to be naturally truncated at the C-terminus of the δ-endotoxins. Removal of a small C-terminal fragment of 11 amino acids causes a loss of the activity of CryIIA δ-endotoxins (Widner & Whiteley 1989). The C-terminus of CryIIIA and CryIVD δ-endotoxins contains a conserved domain that is required for toxicity in several other CryI-activated δ-endotoxins (Caroll et al. 1989, Widner & Whiteley 1989). Degradation of these C-terminal domains will likely result in the loss of insecticidal activity of CryIIIA and CryIVD δ-endotoxins. However, these naturally truncated proteins may undergo protease cleavage in the insect

midgut. A smaller protein of 55 kDa from CryIIIA δ-endotoxin was produced from a 67 kDa δ-endotoxin exposed to the digestive juice of *Tenebrionis molitor* (Slaney et al. 1992). This 55 kDa CryIIIA was fully active. Removal of up to 159 amino acid residues from the N-terminus of CryIIIA δ-endotoxins did not decrease its insecticidal activity (Caroll et al. 1989, McPherson et al. 1988). The role of protease in Coleoptera is still in question (Li et al. 1989, Carroll et al. 1989). *B. thuringiensis* subspecies *tenebrionis* produces the crystal that contains a major polypeptide of 67 kDa and minor polypetides of 73, 72, 55 and 46 kDa (Caroll et al. 1989). During sporulation, the minor polypeptide of 73 kDa decreases, while the concentration of 67 kDa crystal protein increases. The finding that a smaller trypsin treated δ-endotoxin, 55 kDa, is as toxic as the native CryIIIA δ-endotoxin raised the question of the significance of the endogenous protease activity after the crystal release (Carroll et al 1989).

There is extensive variation in the size and structure of the inclusion proteins, the intermediate protoxins, and the active δ-endotoxins. These wide variations are presumed to relate to the insect specificity of the δ-endotoxins. Generally, however, the activated δ-endotoxin is comprised of three structural regions: an N-terminal region, the toxic domain consisting of several conserved hydrophobic regions and a conserved C-terminal region. A variable region between the toxic domain and the conserved C-terminal region contains most of the residue differences (Aronson et al. 1986, Höfte & Whiteley 1989, Choma & Kaplan 1990). The most recent publication of the molecular structure of a δ-endotoxin was on CryIIIA (Li et al. 1991). The 67 kDa CryIIIA δ-endotoxin that aligns with the N-terminal half of 130-140 kDa Cry δ-endotoxins is composed of three distinct domains. Domain I is a bundle of seven amphipathic helices. The α_5 helix is the center of the bundle surrounded by the outer six helices. These outer six helices are comprised of hydrophobic residues on the side facing the α_5.helix The amphipathic helix is a protein domain commonly found in transmembrane pores. Therefore, this domain may facilitate

the membrane insertion and pore formation. Domain II is composed of three β-sheets laid side by side. The non-conserved region of the CryIII δ-endotoxin is mainly part of the domain II. This domain probably determinis the specificity of the receptor binding domain. Residues from segments within domain II, responding to sheets which are responsible for the specificity of the δ-endotoxins, are found also in CryI and CryII δ-endotoxins. Domain III is responsible for strands forming two sheets of the β-sandwich. A high degree of conservation of the C-terminal region of most Cry δ-endotoxins is found in this domain. The buried strands of an inner anti parallel sheet in domain III seem to be the core of C-terminal that are resistant to proteolysis. Beyond structural stability and integrity of the δ-endotoxin, the function of domain III remains unclear. The conserved block 4 in the CryIa δ-endotoxin was observed to be involved in ion conductance indicating a functional role of the domain III (Chen et al. 1993).

All insecticidal proteins of *B. thuringiensis* δ-endotoxins are toxic only after ingestion by the susceptible insect. The sign of intoxication following ingestion of δ-endotoxin is cessation of feeding as a result of paralysis of the gut and mouth parts. In comparison to other microbial δ-endotoxins, this response is extremely rapid, especially when we consider that the crystals have to be dissolved and activated in the gut juice, and the active moiety has to reach the site of action. Subsequent to the paralysis of the gut and mouth, ingested δ-endotoxins are solubilized and, in most cases, proteolyticaly digested to the active form. The activation takes place in the gut. The combined action of alkalinity of gut pH and protease activity are responsible for the dissolution of protoxin.

Histological studies have demonstrated that Cry δ-endotoxins disrupt the midgut epithelium of susceptible larvae (Lüthy & Ebersold 1981, Endo & Nishiitsutsuji-Uwo 1980, Percy & Fast 1983, Bauer & Pankratz 1992). Typical histophatological alteration results in swelling of the cytoplasma, the mitochondria, the Golgi complexes and the endoplasmic reticulum with subsequent loss of their characteristic structure. The content

of mitochondria is dissolved, leaving only a membranous fragment. The nuclei also swells. Vacuolization occurres between the outer and the inner membrane of the nuclei (Lüthy & Ebersold 1981). Subsequently, connections between cell membranes are disrupted resulting in the separation of cells from each other. The cells burst and release their cytoplasmic content into the lumen (Lüthy & Ebersold 1981, Endo & Nishiitsutsuji-Uwo 1980, Bauer & Pankratz 1992).

The δ-endotoxin apparently binds to specific receptors localized on the brush border midgut epithelium and induces pore formation. Binding seems to be an essential step in toxicity. Studies demonstrated a close correlation between binding affinity and toxicity (Endo & Nishiitsutsuji-Uwo 1980, Hofmann & Lüthy 1986, Hofmann et al. 1988a, 1988b, Van Rie et al. 1989, 1990a, 1990b, Ferré et al. 1991, Höfte and Whiteley 1989, Li et al. 1990, Knight et al. 1994). However, more recent studies have demonstrated a degree of nonspecific binding in certain insect species (Garczynski et al. 1991). It appears, in some species, that high affinity binding may occur without killing the insect. Thus binding is necessary, but not sufficient for toxicity.

Binding of CryI δ-endotoxins to the midgut epithelium of European corn borers, *Ostrinia nubilalis*, larva was characterized. Two independent binding receptors were observed to be found in the brush border gut epithelium (Denolf et al. 1993). CryIA(b) and CryIA(c) δ-endotoxins were recognized by the same receptor. A competitive binding study showed that CryIB δ-endotoxin replaced neither CryIA(b) nor CryIA(c) δ-endotoxins. The study indicated a different receptor for the crystal protein exists in the midgut of European corn borers. Different levels of toxicity of the three proteins were observed in bioassay tests. The different toxicities between CryIA(b) and CryIA(c) δ-endotoxins appeared because of affinity differences of those proteins to the same receptor. As demonstrated here, the existence of receptors with different specificities could be a very useful feature for delaying the development of resistance to δ-endotoxins.

The binding study of CryIIIA δ-endotoxin with ^{125}I was done with Colorado potato beetles and Southern corn rootworms, *Diabrotica undecimpunctata howardi* Barber (Slaney et al. 1992). CryIIIA δ-endotoxin showed higher affinity on the midgut brush border of Colorado potato beetles than on Southern corn rootworms. It appears that the susceptibility to δ-endotoxin is associated with the affinity of solubilized protein to the receptors residing in the midgut brush border.

The binding of the δ-endotoxin to the receptors causes a conformational change in domain I (which is an amphipathic helix) so that the hydrophobic residues are in close proximity with the membrane and initiate pore formation. The small pores in the membrane cause an increase in potassium ion permeability of the midgut epithelium. Increased permeability to K^+ would have an effect of decreasing membrane potential and increasing the intercellular pH. The leak of K^+ subsequently is followed by the leakage of water into midgut cells causing the cells to swell and lyse. This mode of activity has been demonstrated in Lepidoptera (Sacchi et al. 1986, Knowles and Ellar 1987, Höfte and Whiteley 1989, English et al. 1991). The disruption of midgut structure and function leads to ion and pH imbalances in the hemolymph, total body paralysis and eventually death. Investigation on CryI δ-endotoxin showed that the δ-endotoxins alter the permeability of lepidopteran midgut apical membranes for monovalent cations (Sacchi et al. 1986, Crawford & Harvey 1988, Wolfersberger 1989, Carroll & Ellar 1993). Changes in the membrane permeability of *Manduca secta* midgut brush-border membrane vesicles after addition of CryI δ-endotoxin was also studied. The permeability of the membrane was significantly affected by CryIA δ-endotoxin. The change was relatively non-selective among solutes. Cations, anions and neutral solutes all traverse the membrane to an increased extent in the presence of CryIA(c) δ-endotoxin. No effect on brush-border membrane vesicles was observed when CryIB δ-endotoxin was used in a similar experiment (Carroll & Ellar 1993).

The biological action of CryIIIA δ-endotoxin has also been studied (Slatin et al. 1990, Li et al. 1991). The ability of CryIIIA δ-endotoxin to induce ion leakage in planar lipid bilayers has been demonstrated (Slatin et al. 1990). The leakage of K^+ and H_2O is probably caused by induced δ-endotoxin pore formation in the plasma membranes. Domain I of the CryIIIA δ-endotoxin has been proposed as the portion of the δ-endotoxin molecule that penetrates the membrane. The membrane might internalize the hydrophobic surfaces of the protein bringing the protein in closer to the membrane. The close contact of the hydrophobic surfaces of the protein with the cell membrane may cause conformational changes in the δ-endotoxin (Li et al. 1991). The conformational changes might be responsible for the formation of the pores or channels in planar lipid bilayers. Consequently, ion flow occurs with eventual vesicle or cell lysis. The pore formation process is thought to be initiated by the interaction of the CryIIIA δ-endotoxin with putative receptors, phospholipids on the surface of the membrane (English et al. 1990, Li et al. 1991, Gazit & Shai 1993).

Bacillus thuringiensis δ-endotoxin: Are We Losing The Promise?

The field applications of *B. thuringiensis* δ-endotoxin in controlling agriculturally important insect pests is just beginning to lead to the selection of resistance insects (McGaughey 1985, Tabashnik et al. 1990, McGaughey & Whalon 1992, Whalon et al. 1993). Yet development of field resistance toward *B. thuringiensis* δ-endotoxin has been slow. Several factors have probably contributed to this delayed evolution of resistance. These may include a limited selection pressure on pest populations due to marginal field efficacy. Intensity of selection pressure in the field has not been very strong because of: 1) limited use of the microbial insecticide, 2) very short residual activity, 3) new plant growth

that does not have residues of *B. thuringiensis* δ-endotoxin, and 4) the reservoir of susceptible genotypes dispersing into sparse fields that would 'flood out' resistant genotypes (Stone et al. 1989, McGaughey & Beeman 1988).

Insects do have, however, the capacity to develop resistance to *B. thuringiensis* δ-endotoxin. A few important insect pests from orders Diptera, Lepidoptera and Coleoptera were reported to develop resistance. Harvey and Howell (1965) reported a selection with *B. thuringiensis* δ-endotoxin on a laboratory colony of house flies, *Musca domestica* L. After continuous laboratory selection for 50 generations, the colony became resistant. The resistance was not stable, however, and it appeared to decline during 20 subsequent generations without selection.

Resistance to *B. thuringiensis* δ-endotoxin in *Drosophila melanogaster* was obtained in the laboratory (Carlberg & Lindstorm 1987). Laboratory colonies of fruit flies were used to start the selection. At least fivefold resistance in 70 generations to the δ-endotoxin of the *B. thuringiensis* serotype H-1 was observed in the fruit fly colonies. Another dipteran, *Aedes aegypti*, was reported to develop resistance to δ-endotoxin produced by *B. thuringiensis* subspecies *israelensis*. The mosquito strains were raised in the laboratory for five generations before being used to start the selection. After fourteen generations of selection, a small but statistically significant increase in resistance was observed (Goldman et al. 1986).

An almond moth (*Cadra vautella* (Walker)) colony was started from an infested bin in southern Texas and reared in the laboratory for at least 130 generations before being used for selection (McGaughey & Beeman 1988). Selection was done by exposing the neonates with *B. thuringiensis* subspecies *kurstaki* δ-endotoxin incorporated in their diet. Resistance increased seven-fold after 21 generations of intensive selection. A sunflower moth (*Homoseosoma electellum* (Hulst)) colony, maintained on a wheat germ-based diet for at least three years, was used for selection with *B. thuringiensis* subspecies *kurstaki* δ-endotoxin (Brewer 1991). A newly hatched larva was placed on a diet topically treated

with suspension of the δ-endotoxin. A significantly higher tolerance to the δ-endotoxin was first observed in generation eight.

A major lepidopterous pest on cotton, *Spodoptera littoralis* (Boisduval), was subjected to a laboratory selection (Salama & Matter 1991). The cotton leafworm was raised in the laboratory for several years before being selected with δ-endotoxin from *B. thuringiensis* subspecies *kurstaki* HD-1 (Dipel). After eight generations of selection, the colony was significantly more resistant than the unselected one. Another Lepidoptera, *Choristoneura fumiferana* (L.), was observed to develop resistance (< ten fold resistance) after laboratory selection for eight generations (see Tabashnik 1994).

Laboratory selection of a stored grain pest resulted in resistance to *B. thuringiensis* subspecies *kurstaki* δ-endotoxin(McGaughey 1985). Larvae or pupae of *Plodia interpunctella* were collected from bins of *B. thuringiensis* δ-endotoxin-treated grain and had been maintained for 16-26 generations before the selection studies began. The colonies were subcultured for several successive generations on a *B. thuringiensis* δ-endotoxin-treated diet. The diet contained the δ-endotoxin at concentrations that caused 70-90% larval mortality. Within three generations, the LC_{50} of one of the colony was 29 times higher than the unselected colony. The level of resistance increased to more than 100 fold in sixteen generations. When selection pressure was continued for another 20 generations, the level of resistance increased to greater than 250 fold relative to the unselected parent colony (McGaughey & Beeman 1988).

Laboratory colonies of tobacco budworms, *Helicoverpa virescens* (F.), in which wild males were introduced annually, were used for selection. A genetically engineered *Pseudomonas fluorescens* expressing CryI δ-endotoxin was used to select the neonates. The colony was reared for three generations prior to subculturing the neonates on a δ-endotoxin-treated diet. Significant tolerance was first observed at generation three. By generation seven, the level of resistance increased to 24 fold (Stone et al. 1989). Different populations of tobacco budworm were selected against *B. thuringiensis* δ-endotoxin

(Gould et al. 1992). A fourth generation colony was used to start the selection with a CryIA(c) treated diet. After ten generations, the level of resistance was about ten fold. The resistance ratio between selected and unselected colonies increased to 50 fold after seventeen generations of selection. Genetic analysis of this strain indicated that the resistance was inherited as a partially recessive trait. Further study showed that this particular colony exhibited cross-resistance to *B. thuringiensis* δ-endotoxins (CryIA(b), CryIIA).

There is very little information on *B. thuringiensis* δ-endotoxin resistance in field populations. The only documented species showing resistance to *B. thuringiensis* δ-endotoxin in a field population was the diamondback moth, *Plutella xylostella* (L.) (Tabashnik et al. 1990, 1991 1992). Tabashnik et al. (1990) reported results from a survey of responses of diamondback moths collected from commercial fields of watercress, cabbage, and broccoli in Hawaii. In a laboratory bioassay, diamondback moths collected from watercress fields intensively treated with *B. thuringiensis* subspecies *kurstaki* δ-endotoxin were observed to be 25 times more resistant than the susceptible laboratory colonies. This field population was further selected in the laboratory. After nine generations of selection, the resistance level was significantly greater (up to 36 fold resistance) than the susceptible laboratory colonies (Tabashnik et al. 1991). Rapid development of resistance may be a result of heavy treatment with *B. thuringiensis* subspecies *kurstaki* δ-endotoxin on field populations of diamondback moths. Various crosses between selected and unselected colonies were conducted to determine the mode of inheritance of the resistance (Tabashnik et al. 1992). Responses of the progeny from reciprocal crosses were not different. The results indicate an autosomal inheritance. The LC_{50}s of the F_1 offsprings were not significantly greater than LC_{50} of the unselected colony. This result showed that the resistance in diamondback moths is inherited as a recessive trait. Further field survey of resistance in diamondback moths was done in six

states of the United States and in Indonesia (Shelton et al. 1993). This survey concluded that intensive use of *B. thuringiensis* δ-endotoxin increases the resistance problems.

Several theories on possible physiological mechanisms of *B. thuringiensis* δ-endotoxins resistance are suggested. Most of the studies have been done on lepidopteran species (Endo & Nishiitsutsuji-Uwo 1980, Sacchi et al. 1986, Knowles & Ellar 1987, Höfte & Whiteley 1989). Altered binding affinity is one of the hypothesized mechanisms of insect resistance to *B. thuringiensis* δ-endotoxin. A study on *P. interpunctella* showed evidence that resistance to *B. thuringiensis* CryI δ-endotoxin is due to a change in binding affinity of receptors or alteration of the binding sites (Van Rie et al. 1990). Resistance to CryIA(b) δ-endotoxin is associated with the reduction of the affinity of the receptors for δ-endotoxin. Furthermore, resistant *P. interpunctella* gained sensitivity to CryIC δ-endotoxin. Changes in affinity of the receptors to δ-endotoxins have also been observed in the resistance of *H. virescens* (Gould et al. 1992). However, a different population of *H. virescens* (MacIntosh et al. 1991) showed the contrary result. No significant difference in CryIA(b) δ-endotoxin binding affinity between selected and unselected strains was found. Furthermore, cross-resistance with CryIA(c) δ-endotoxin was observed in these strains suggesting that a different mechanism of resistance may be involved.

A study was conducted to determine how changes in protease inhibitors would affect the proteolytic processing of the δ-endotoxin. Very low effects of the inhibitors on response of *P. xylostella* (both resistant and susceptible strains) to *B. thuringiensis* δ-endotoxin suggested that altered proteolytic processing was not a major mechanism of resistance (Tabashnik et al. 1992). Possible behavioral avoidance of formulated and/or purified *B. thuringiensis* δ-endotoxin has been reported in *P. xylostella* (Gould & Anderson 1991, Scwartz et al. 1991). The laboratory choice tests suggested that there is no evidence for behavioral resistance.

The complex mode of action of CryIIIA δ-endotoxin suggests the possible mechanisms of resistance in Colorado potato beetles, including a decrease in δ-endotoxin solubilization (assuming that protease is critical for CryIII δ-endotoxin solubilization), conformation changes of solubilized protein in the insect gut, membrane turnover, and recovery.

Thesis Objectives

The developments of insect-resistant plants would provide more effective, less costly, and more environmentally attractive pest control. This process has been successfully accomplished through the *Agrobacterium tumefaciens* binary vector system. The field expressions of the δ-endotoxin genes in transgenic plants have also been demonstrated to be effective in suppressing insect pest populations (Adang et al. 1987, Fischhoff et al. 1987, Vaeck et al. 1987, Delannay et al. 1989). This progress raised growing concern about the durability of *B. thuringiensis* δ-endotoxin (McGaughey & Whalon 1992). The ability of several major insect pests to tolerate *B. thuringiensis* δ-endotoxin has been demonstrated in laboratories. Therefore, insect resistance will be a critically important consideration as *B. thuringiensis* δ-endotoxin applications (transgenic plant releases or conventional δ-endotoxin sprays) increase. Without a cautious and wise resistance management program, the loss of the effectiveness of *B. thuringiensis* δ-endotoxin will occur in only a few years.

Resistance management is any attempt to prevent or delay adaptation of insect pests to toxic agents. The strategy in managing pest resistance is to maintain resistant alleles at very low frequencies. This could be achieved by preserving a sufficient population of susceptible individuals or alleles, and by reducing the rate and probability of resistance development. Tactics to implement these strategies might include: 1) variation

in dose or rate of insecticide applications, 2) reducing the frequency of applications, 3) simultaneous use of two or more chemical with different modes of action or target site, 4) rotation, alteration or sequential applications of insecticides with different modes of action or target sites.

Eventually, resistance in Colorado potato beetle populations will undoubtedly develop in the field, when conventional *B. thuringiensis* δ-endotoxin and transgenic *B. thuringiensis* δ-endotoxin plants are used widely on potato, tomato, egg plants and other hosts. Therefore, understanding of *B. thuringiensis* δ-endotoxin resistance is critically important to developing strategies for managing resistance. The goal of this action should be to reduce the selection pressure of this bioinsecticide and to prolong the utility of this environmentally safe bioinsecticide (Croft 1990). The ideal strategy is to develop resistance management as early as possible, or even before the resistance has evolved in field population of the targeted insect. Alternative controls are urgently needed to extend the effectiveness of bioinsecticides. Applications of these insecticides to selectively manage resistance development is an obvious necessity.

Resistance monitoring is one of the key features of any resistance management programs. An appropriate monitoring system should provide for early detection of resistance as it begins in the field. This initial warning will allow resistance managers the time to take necessary steps to abate resistance evolution.

In 1987, Whalon et al. (1993) initiated field selection of Colorado potato beetle with CryIIIA δ-endotoxin of *Bacillus thuringiensis*, and subsequently selected them in the laboratory resulting in over 200 fold resistance. Understanding the mode of inheritance of this resistance phenotype in a Colorado potato beetle population is critical to in-depth knowledge of the resistance. The management of this resistance will depend on whether the resistance is inherited in a discrete manner or as a continuously quantitative trait. When the resistance is a mono- or oligogenic trait, the number of alleles at the resistance

determining loci and the dominance relationships among these alleles should be determined (Curtis et al. 1978). The identification of interactions between genes conferring resistance as well as the modifying loci are an important step in understanding the evolution of insecticide resistance (Uyenoyama 1986). It is particularly critical to know the mean levels of resistance, the phenotypic variances and the additive or non-additive genetic variance (Via 1986). This study was done to characterize the *B. thuringiensis* δ-endotoxin resistance in Colorado potato beetles, and to apply the knowledge gained to developing a strategy of early prevention of resistance evolution in field populations.

Biotechnology has provided techniques for qualitative and quantitative detection of insecticide resistance. These techniques allow researchers to determine polymorphism in insecticide resistance within a species, and also among different species in different populations. Nearly five years ago, a new genetic assay was developed by two different laboratories (William et al. 1990, Welsh & McClelland 1990). This technique, Random Amplified Polymorphic DNA (RAPD), detects nucleotide sequence polymorphism in the genome of a wide variety of different species. A small amount of genetic variation is revealed by amplification of certain regions of the genomic DNA. The genomic DNA, as a template, is subjected to amplification using the polymerase chain reaction (PCR) system and RAPD oligonucleotide primers. This technique makes use of a single oligonucleotide primer (usually ten base pairs in length) that hybridizes and amplifies arbitrary regions of a genome. With these advanced molecular techniques, DNA-based genetic fingerprinting of numerous species has already been reported (Black IV et al. 1992, Landry et al. 1993). Two years after the original invention, a new adoption of the RAPD technique was developed (Liang & Pardee 1992) and was called Reverse Transcription-PCR (RT-PCR). The procedure uses an additional primer that will anchor the poly(A) tail at the 3' end of total mRNA. The cDNA resulting from this amplification is then used as a template for subsequent RAPD assays. Developing genetic markers for gene(s) conferring resistance to *B. thuringiensis* δ-endotoxin in Colorado potato beetles will lead to further

understanding of the inheritance of resistance. Furthermore, the markers developed will be very useful tools for early detection or monitoring of the development of resistance in field populations.

The objectives of this research were:

1) to examine the continued selection of Colorado potato beetle with *B. thuringiensis* δ-endotoxin CryIIIA,
2) to identify the mode of inheritance of resistance to *B. thuringiensis* δ-endotoxin CryIIIA in Colorado potato beetle,
3) to determine the heritability of resistance in Colorado potato beetle selected with the *B. thuringiensis* δ-endotoxin CryIIIA, and
4) to identify markers that can serve as diagnostic probes for *B. thuringiensis* δ-endotoxin resistance in Colorado potato beetle field populations.

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CHAPTER I

Inheritance of Resistance to *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA δ-endotoxin in Colorado Potato Beetle (Coleoptera: Chrysomelidae)

INTRODUCTION

Coleopteran specific δ-endotoxin, CryIIIA (Hofte & Whiteley 1989), a product of *Bacillus thuringiensis* subsp. *tenebrionis* (Krieg et al. 1983, 1984; Sekar et al. 1987; McPherson et al. 1988), has been formulated into several commercial insecticides (Ferro & Gelernter 1989, MacIntosh et al. 1990) that are active on the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and have been genetically engineered into potatoes (Gasser & Fraley 1989, Brunke & Meeusen 1991). This δ-endotoxin of *B. thuringiensis* appears to be an environmentally sound alternative to broad spectrum synthetic organic insecticides for controlling Colorado potato beetle, the most destructive pest on potatoes. Recent development of Colorado potato beetle resistance to synthetic organic insecticides in the eastern and midwestern United States (Ionnidis et al. 1991) have contributed to an increase in the use of *B. thuringiensis* CryIIIA δ-endotoxin.

For > 20 yr, various *B. thuringiensis* isolates have been used commercially to control insect pests. However, one species has developed resistance in the field (Tabashnik et al. 1990). Resistance in the tobacco budworm, *Heliothis virescens* (F.), and Colorado potato beetle (Whalon et al. 1993) has been reported only from laboratory selection experiments, but the capacity for resistance in these species is of concern because of the great economic significance of these two pest species. The recent discovery that several important species of pest insects can develop resistance to *B. thuringiensis* δ-endotoxins now raises concerns regarding the long-term use of this biological insecticide in pest control. Questions of longevity are especially critical in the rapidly advancing area of plant genetic transformation, which has emphasized the use of *B. thuringiensis* δ-endotoxin genes to impart pest resistance in several major crop species (Gasser & Fraley 1989, Boulter et al. 1990, Brunke & Meeusen 1991). Regardless of how the *B.*

thuringiensis δ-endotoxins is delivered, insect pests might develop tolerance. The intensive application of *B. thuringiensis* δ-endotoxin through conventional sprays or transgenic plants deployment could seriously diminish the economic value of this technological development and force continued reliance on other chemical insecticides (Gould 1988a, b; Stone et al. 1989; Tabashnik et al. 1992).

The genetic basis of laboratory-selected resistance to *B. thuringiensis* δ-endotoxin was examined in diamondback moth, *Plutella xylostella* (L.) (Tabashnik et al. 1992) and Indianmeal moth, *P. interpunctella* (Hübner) (McGaughey 1985, McGaughey & Beeman 1988). This genetic knowledge may be useful in devising strategies to slow resistance evolution. However, inheritance of laboratory-selected resistance may differ from resistance selected in the field (Roush & McKenzie 1987, Tabashnik et al. 1992).

Beginning in 1987, we selected a field and laboratory colony of Colorado potato beetle with the CryIIIA δ-endotoxin of *B. thuringiensis* (Whalon et al. 1993). The objective of the study reported here was to examine and report the continued selection and the inheritance of resistance.

MATERIALS AND METHODS

CryIIIA δ-Endotoxin Sources

M-One insecticide (AI: *B. thuringiensis* subsp. *tenebrionis* δ-endotoxin, Mycogen, San Diego, CA) containing 40,900 Colorado potato beetle International Units (CPB IU)/mg of formulation and 8,800 ± 10% μg (mean ± SEM) CryIIIA δ-endotoxin per milliliter of formulation was initially used for selection and bioassays. Spud-Cap Bioinsecticide (AI: *B. thuringiensis* subsp. *tenebrionis* δ-endotoxin encapsulated in killed *Pseudomonas florescens* cells, Mycogen, San Diego, CA) was used beginning in June 1989. Spud-Cap contained 83,400 CPB IU/mg formulation and 12,300 + 10% μg (mean ± SEM) CryIIIA δ-endotoxin per milliliter of formulation.

Beetle Strains, Rearing, and Selection

Strains of Colorado potato beetle used for the experiment were maintained in our laboratory at 25 ± 2°C and a photoperiod of 16:8 (L:D) h. The origin and maintenance of both susceptible and resistance strains were described earlier by Whalon et al. (1993). Susceptible and resistant strains were from the same stock collected in 1987 and 1988 from seven counties in Michigan. Second instars (3 d old) were selected in every generation at a level of CryIIIA δ-endotoxin concentration that prevented >98% from reaching the adult stage. Beetles were exposed for 2 - 3 d on 'Superior' potato foliage treated with a CryIIIA δ-endotoxin concentration of 1,500 - 741,000 µg/liter of water (Whalon et al. 1993).

To select beetles, potato petioles (5 leaflets) were inserted into 2-ml vials filled with water, dipped five times into the CryIIIA δ-endotoxin solution, and allowed to air dry before being transferred to individual petri-dishes (15 cm diameter). Twenty larvae were placed on each leaf and held at 25 ± 2°C and a photoperiod of 16:8 (L:D) h in a growth chamber. Foliage was checked daily and water was replenished as needed. Larvae were placed on the foliage within 30 min after the CryIIIA δ-endotoxin application had air dried; they were allowed to move and feed freely. After exposure to CryIIIA δ-endotoxin, surviving larvae were transferred to untreated foliage of potted potato plants within rearing cages for 5 - 10 d until pupation.

Pair-by-pair cultures were used to increase homogeneity for genetic analysis of the strains. The sex of newly emerged adults from selections was determined; they were paired before being placed on potted potato plants covered with a cage made of a 2-liter soda bottle. Progeny from each pair of resistant strains was selected and maintained separately for five generations before genetic analysis.

Inheritance and Stability Studies

Resistance dominance, sex linkage, and chromosomal inheritance were determined by performing reciprocal crosses as pair by pair matings between susceptible and resistant individuals. Investigations were done with G_{17} - G_{34} beetles. Newly emerged virgin females and males of G_{29} of each strain were separated until use. One male and one female were paired on caged potato plants. Progeny (F_1) from each pair of each cross (S × R and R × S) was tested. A portion of the F_1 progeny was reared to maturity for further experimentation. The experiment was done with nine pairs for reciprocal crosses. Four pairs of backcrosses between adult F_1 and susceptible parent were done to determine whether resistance was inherited as a monogenic or polygenic trait. We tested the hypothesis that the resistance was inherited by a single dominant gene. To test this hypothesis, we calculated the expected mortality of the F_2 at concentration (c) as (percentage of expected mortality of F_1 at c + percentage of expected mortality of suceptible at c)/2. Mortality from each backcross was observed; chi-square analysis was used to compare observed mortality with mortality expected assuming simple Mendelian inheritance.

Larvae from the G_{17} generation were separated from the selected colony and maintained for seventeen generations as described above on untreated potato plants in 2-liter cages. Second instars in each generation were tested to determine the stability of the resistance.

Bioassays

Activity of CryIIIA δ-endotoxin on Colorado potato beetle was assessed with 3 d old larvae. Five or six serial dilutions of CryIIIA δ-endotoxin were made to give concentrations ranging from 0.125 to 16 times the expected LC_{50} (Whalon et al. 1993). Potato foliage was trimmed to the terminal five leaflets and the petiole was inserted into a 2-ml vial containing water. Each petiole was dipped five times into one of the CryIIIA δ-

endotoxin solutions and allowed to air dry before being placed in a 15-cm petri-dish. At least five petioles were prepared per concentration; we used no more than 10 larvae on each leaf at each concentration and at least 30 larvae per concentration. Larvae were held at 25 ± 2°C, 50 - 60% RH, and a photoperiod of 16:8 (L:D) h in a growth chamber. Mortality was assessed 96 h after treatment.

Data Analysis

Data were analyzed by probit regression (Finney 1971); Abbott's (1925) formula was used to correct for control mortality. LC_{50}s were compared between generations to monitor the progress of resistance. Failure of 95% CL to overlap was used as the criterion for significant differences at LC_{50} ($P < 0.05$). If confidence limits did not overlap, the LC_{50} values were considered to be significantly different. The resistance ratio was calculated by dividing the LC_{50} of the selected with the LC_{50} of the unselected strain within each generation. Concentration - mortality relationships obtained for F_1 crosses were used to determine the autosomal or sex-linked nature of resistance inheritance. The dominance level (D) of CryIIIA δ-endotoxin resistance in F_1 progeny was estimated with the index given by Stone (1968).

$$D = \frac{2 X_b - X_a - X_c}{X_a - X_c}$$

(where $X_a = \log_{10}(LC_{50})$ of the resistant colony, $X_b = \log_{10}(LC_{50})$ of the heterozygous colony, and $X_c = \log_{10}(LC_{50})$ of the susceptible colony). This formula will result in a value of -1 if resistance is completely recessive, a value of 0 if there is no dominance, and a value of +1 if resistance is completely dominant. The standard error was estimated by taking the square root of variance of D (Preisler et al. 1990):

$$\sigma_D = \frac{4}{(X_c - X_a)^2}\left\{ \sigma_{X_b} + \frac{(X_b - X_c)^2}{(X_a - X_c)^2}\sigma_{X_a} + \frac{(X_b - X_a)^2}{(X_a - X_c)^2}\sigma_{X_c} \right\}$$

The D is significantly different from ±1 when the approximate 95% CL value (D ± 2 SEM) includes ±1.

Chi-square values from observed and expected mortality of the F_1 backcrossed progeny were calculated to determined the monofactorial inheritance of resistance. Expected mortality was estimated assuming simple Mendelian inheritance, where backcross mortality of any given dose was the sum of half of the mortality of the parental strains. The chi-square test for goodness-of-fit for expected mortalities was calculated with formulas obtained from Finney (1971).

RESULTS

Resistance Selection

Table I.1 shows the progression of resistance from the F_{13} through F_{29} generations under selection with CryIIIA δ-endotoxin. Significantly higher LC_{50}s than the susceptible colony were observed in generations F_{17} and F_{21}. A 3-fold increase in resistance ratio was observed from the F_{13} to the F_{29}; this increase represented a >200-fold difference between the susceptible and resistant strains. The LC_{95} of F_{13}, F_{16} and F_{29} were significantly different from LC_{95} of the F_{15}, F_{17}, and F_{21}, respectively. The unselected strain, which was also tested throughout as a control, had LC_{50}s that did not vary significantly from F_{13} to F_{34} (1.69 ± 0.20 mg CryIIIA δ-endotoxin per liter of water).

Resistance Inheritance

The probit regression statistics of the parental strains and their F_1 progeny (Table I.2) indicated no significant differences in lethal concentrations were observed between the two reciprocal F_1 generation crosses.

Table I.1. Progression of resistance in Colorado potato beetle to the CryIIIA δ-endotoxin of *Bacillus thuringiensis*.

Generation[a]	*n*	Slope± SEM	LC_{50}[b] (95% CL)	RR[c]	χ^2	df
G_{13}	780	1.27±0.07	133a (111 - 160)	82	2.32	3
UN_{13}	240	1.92±0.29	1.62 (1.22 - 2.19)		2.77	5
G_{15}	480	1.07±0.07	162a (125 - 215)	98	1.33	3
UN_{15}	240	1.19±0.11	1.66 (1.18 - 2.52)		2.72	5
G_{16}	480	1.86±0.16	241ab (204 - 291)	146	2.33	3
UN_{16}	240	1.69±0.11	1.65 (1.25 - 2.20)		0.57	5
G_{17}	480	1.16±0.09	330bc (263 - 440)	223	2.48	3
UN_{17}	240	1.46±0.09	1.49 (1.11 - 2.08)		2.00	5
G_{21}	540	0.95±0.07	369bc (286 - 535)	222	0.31	3
UN_{21}	240	1.90±0.29	1.66 (1.25 - 2.27)		2.32	5
G_{29}	168	2.30±0.12	484c (379 - 625)	293	1.16	3
UN_{29}	192	1.29±0.08	1.65 (1.11 - 2.42)		1.03	5

Values followed by the same letter are not significantly different if their 95% CL between generations of resistance colonies overlap.

[a] Filial generation. G_n resistant strain generation n; UN_n unselected strain generation n

[b] mg CryIIIA δ-endotoxin per liter of water.

[c] Resistance ratio = LC_{50} of $G_n \div LC_{50}$ of UN_n

Table I.2. Response of susceptible and resistant Colorado potato beetle and the genetic crosses to the CryIIIA δ-endotoxin

Strain and Cross[a]	*n*	Slope±SEM	LC_{50}[b] (95% CL)	RR[c]	χ^2	df
Resistant parent	168	2.30±0.12	484a (379 - 625)	293	1.16	3
$F_1(R_f \times S_m)$	288	2.10±0.90	255b (208 - 318)	154	7.89	9
$F_1(S_f \times R_m)$	300	1.87±0.16	245b (191 - 314)	148	8.58	10

Values followed by the same letter are not significantly different if their 95% CL overlap.

[a] S, susceptible; R, resistant; f, female; and m, male.

[b] mg CryIIIA δ-endotoxin per liter of water.

[c] Resistance ratio = LC_{50} of resistant parent or F_1 + LC_{50} susceptible strain.

In Colorado potato beetle, we thus concluded that resistance to CryIIIA δ-endotoxin is autosomaly inherited and that there is no maternal influence (assuming that the mode of sex determination is XY, XX). Approximately the same degree of dominance values (D ± SEM) were observed in these F_1 crosses (R × S and S × R), which were 0.77 ± 0.06 and 0.76 ± 0.059, respectively. These values were significantly different from -1, indicating that the resistance trait was not completely recessive. The D values ranged from 0.66 to 0.89 and 0.64 to 0.87 respectively indicating that resistance might be inherited through incompletely dominant gene(s).

Mortality of backcross progeny with susceptible parents deviated from expected mortalities. The observed mortality were higher at all concentrations (Table I.3). The test of monogenic model showed no significant deviation between observed and expected mortality at three of six concentrations. Unlike other studies (Halliday & Georghiou 1985, Roush et al. 1986), significant deviation ($P < 0.01$, df = 1) from expected values were produced at low concentrations (≤29.64 mg/liter). Deviations from expected mortality in the backcross experiment suggested that more than one locus may be responsible for resistance.

Resistance Stability

After selection pressure with CryIIIA δ-endotoxin was relaxed, we noticed a downward trend in the resistance ratio. The resistance ratio decreased from 200 to 48-fold in >10 generations. The LC_{50} decreased significantly five generations after the selection pressure was removed (Table I.4). When the colony was continously raised without selection with δ-endotoxin for another 12 generations (G_{22} - G_{34}), the resistance ratio declined within a range of 48 - 81 fold. The LC_{50} of G_{22} - G_{34} were significantly lower than G_{17}. Except for F_{29}, the 95% CL of the LC_{50}s of those generations overlapped. The last generation tested (G_{34}) showed significantly higher tolerance to the δ-endotoxin than the unselected strain.

Table I.3. Chi-square analysis of mortality statistics from F_1 × susceptible backcrosses of Colorado potato beetle (test monogenic model)

Concentration	% Mortality			
mg/liter	Expected	Observed	χ^2	P^a
463.125	97	98	1.29	0.28
185.25	86	95	3.14	0.08
74.10	71	79	2.98	0.09
29.64	46	60	7.74	<0.05*
11.85	22	44	23.73	<0.01*
4.74	9	24	32.35	<0.01*

[a] Values followed by * are significantly different at $P \leq 0.05$

Table I.4. Resistance stability to CryIIIA δ-endotoxin in Colorado potato beetle

Generation[a]	*n*	Slope±SEM	LC_{50}(95% CL)[b]	RR[c]	χ^2	df
G_{17}	240	1.16±0.09	330a (263 - 440)	223	2.48	3
UN_{17}	240	1.46±0.09	1.49 (1.11 - 2.08)		2.00	5
G_{19}	240	0.83±0.06	230ab (155 - 410)	140	0.82	4
UN_{19}	240	1.27±0.09	1.62 (1.16 - 2.33)		0.31	5
G_{22}	210	0.75±0.07	100bc (65 - 186)	60	1.83	3
UN_{22}	240	2.12±0.43	1.67 (1.29 - 2.23)		5.30	5
G_{23}	210	1.36±0.14	134bc (98 - 202)	81	0.97	4
UN_{23}	210	1.60±0.16	1.65 (1.18 - 2.22)		0.37	4
G_{29}	240	2.17±0.47	79c (64 - 102)	48	1.11	3
UN_{29}	192	1.29±0.08	1.65 (1.11 - 2.42)		1.03	5
G_{34}	168	3.25±0.64	121bc (98 - 158)	55	2.77	4
UN_{34}	320	2.92±0.49	2.21 (1.86 - 2.65)		4.15	5

Values followed by the same letter are not significantly different if their 95% CL overlap.

[a] G_n: generation n; UN_n: unselected generation n. The selection was relaxed in the G_{17} generation for seventeen generations (G_{34}).

[b] mg CryIIIA δ-endotoxin per liter of water.

[c] Resistance ratio = LC_{50} of $F_x \div LC_{50}$ unselected strain.

DISCUSSION

A graph of the percentage response (mortality) against a stimulus (toxin) will give a steadily rising curve. However the rate of increase in mortality per unit increase in toxin concentrations is frequently very low in the region of zero or 100% mortality, but higher in the intermediate region so that the curve is actually sigmoidal. When the toxin concentration is measured in metametrix units (log concentration), the curve takes a characteristic normal sigmoid curve. The transformation of percent mortality into probit values also helps to linearize the normal sigmoid curve. In some situations, however, the correlation between the probit values and log concentration is more complicated than previously mentioned.

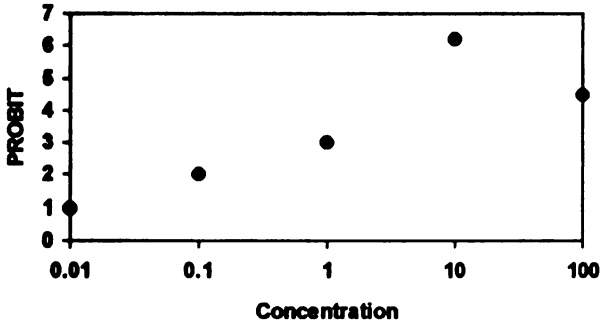

Figure I. 1. Probit values were plotted against concentration of a toxic agent (log concentration).

Some of the transformed data gave plots as shown in the figure above. The more concentrated the toxin, the less mortality was observed. One explanation could be the dimerization or polymerization of the crystal protein. As a consequence, less toxin would have been active to cause disruption; thus less mortality would have resulted.

Previous genetic and biochemical studies of *B. thuringiensis* δ-endotoxin indicate that resistance in insects is commonly inherited as a partially or fully recessive trait (Gould et al. 1992). Study of CryIIIA δ-endotoxin resistance on Colorado potato beetle indicates a different conclusion. Our results suggest that an incomplete dominant gene(s) is involved in CryIIIA δ-endotoxin resistance in Colorado potato beetle. Bioassays of reciprocal crosses produced lines that were close to those of resistant parents. When incomplete or partial dominance exists, at least two different gene interactions can occur. The presence of other genes may hide the effect of the heterozygote resistance gene(s). In addition, epistasis can occur as a consequence of increased or decreased enzyme activities, or changes in pH that effect a particular phenotype (Strickberger 1985). Epistasis could increase the ability of the insect to tolerate the toxic agent by, for example, changing receptor-ligand kinetics, or changing the gut acidity or physiology. Crow (1957) suggested that epistatic interactions can evolve under close inbreeding. This explanation pertain to our selection process with Colorado potato beetle, i.e. a simple relationship between genes in which each makes a contribution to the resistance character. Thus, the introduction of genes from the susceptible to the resistant colony would dilute the effects of these genes. Production of heterozygotes also may result in reduced fitness because of interference between detoxification pathways, binding sites, or normal metabolic processes (Uyenoyama 1986).

The results presented here may indicate that resistance to δ-endotoxin segregates in non monofactorial fashion in backcrosses to susceptible parents. The bioassay of backcross progeny did not produce evidence of monofactorial segregation. When the backcross progeny were exposed to the discriminating concentration (≈60 mg CryIIIA δ-endotoxin per liter of water, which was expected to kill 100% of the susceptible larvae), >50% mortality was observed. These results also suggested that a genetic network controls the resistance mechanism. In the backcross experiments to the susceptible strain, we observed a significantly higher mortality than expected over the three lower

concentrations (≤29.64 mg/liter). With the lower concentrations, the larvae may have continued feeding on the treated leaves longer, resulting in more mortality. We have observed this phenomena is subsequent bioassays of feeding behavior. Loci for genes conferring CryIIIA δ-endotoxin resistance might also be separately segregated.

Our resistance stability experiment demonstrated that the resistance level significantly declined over a five generation period when the selection pressure was removed. However, continued breeding for another 12 generations without selection did not produce further reduction in resistance level. Because further breeding did not produce further reduction in level of resistance, homozygosity apparently had been reached for the majority of the genes determining resistance. The effect of continued inbreeding, as occurs in many laboratory colonies, might cause the reduction of heterozygosity up to certain level that further reduction does not occur. Reintroduction of the susceptible genes may result in further reduction of resistance level. Our results suggest that resistance management of Colorado potato beetle (Whalon et al. 1993) may be possible in potato production if resistance management begins before resistance factors are fixed in targeted populations.

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CHAPTER II

Heritability Estimation of Resistance to *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA δ-endotoxin in Colorado Potato Beetle (Coleoptera: Chrysomelidae)

INTRODUCTION

The potential development of field resistance to pesticides depends on genetic and biological characteristics of the pests as well as the environmental characteristics of the particular population (Keiding 1986). When selection pressure is applied on a particular population, the frequency of the particular genes conferring the resistant trait will change. The change in the proportion of resistance, therefore, is due to genotypic change resulting from selection.

For a given trait, selection gain can be predicted for several initial generations of selection. As the selection progresses, however, the gains are being generally below those anticipated. Eventually the selection brings no response, and 'plateau' is reached (Dobzhansky 1970). Reproductive advantage may affect the selection gain. Gain under selection is determined by the selection advantage of the desired genotype and the reproductive potential of the population.

Any gain in laboratory selections does not represent the response of the population in nature under equal selection pressure. The following are several factors that may contribute to the different results between field and laboratory selections. The rare resistance genes and additional genes may be missing in a laboratory colony as the gene pool is smaller. Furthermore, effects of inbreeding also become more pronounced under intense laboratory selection, because the size of the breeding population is greatly reduced. The difference in response often result in lower mortality in laboratory colony. In a laboratory colony, an artificial selection may result in exploitation of polygenetic variance. On the other hand, field selection tends to act on alleles of single resistance genes (Keiding 1986).

Laboratory selections have been conducted under many different environmental conditions to gain more understanding about resistance development. However, there are some limitations in predicting the progress of selection. In some cases, environmental factors may have significant influence on the response of the individuals in a population toward the selection pressure. In nature, selection may operate much more strongly in favor of individuals able to survive for themselves and overcome the unexpected environmental stress. A fixed and constant environmental condition of artificial selection may decrease the chance survive of individuals with disadvantage environmental condition.

The study of inherited factors uncovered by manipulating laboratory colonies may provide information about the nature of the alleles conferring resistance and their anticipated fate in the population. Laboratory selection for resistance, despite its limitations, is usually included as one element in predicting the probability of pesticide resistance development. The selection gain can be predicted from the heritability of the selected phenotype from each generation (Dobzhansky 1970). Heritability is defined as the ratio of additive genetic variance to phenotypic variance (Falconer 1989). The value of the heritability thus represents the overall genetic inheritance characteristics of a given population. Information collected from artificial selections can be used to estimate the heritability of the traits being selected. Heritability determined from generation to generation of artificial selection can be useful in predicting the potential of resistance development in other populations, especially if the inheritance of genes for resistance is known and markers to detect the presence of the gene(s) in the field population are available. Although no research has yet demonstrated a clear linkage between field population monitoring and laboratory heritability, this is a long-term goal of our laboratory.

In 1987 we initiated field selection of Colorado potato beetle with CryIIIA δ-endotoxin of *Bacillus thuringiensis* (Whalon et al. 1993), and subsequently selected them in the laboratory which resulted in over 200 fold increase in resistance (Rahardja &

Whalon 1995). We report here the estimation of realized heritability from each generation of Colorado potato beetle, *Leptinotarsa decemlineata* (Say), artificially selected with *B. thuringiensis* CryIIIA δ-endotoxin.

MATERIALS AND METHODS

Insect Rearing and Selection

Colorado potato beetle strains (susceptible and resistant) used for this study were reared and selected under conditions as described elsewhere (Whalon et al. 1993, Rahardja & Whalon 1995). Second-instar Colorado potato beetles were selected by exposure for 96 h on potato foliage dipped in a *B. thuringiensis* δ-endotoxin concentration of 1.5-741 mg/liter of water. Twenty larvae were placed on each treated potato leaf and held at 25 ± 2 °C, 50-60% RH and a photoperiod of 16:8 (L:D) h in a growth chamber. After exposure to CryIIIA δ-endotoxin, surviving larvae were transferred to untreated fresh leaves in clean petridishes and maintained until third-instar. The larvae then were placed on potted untreated potato plants within rearing cages until pupation. The percentage of surviving adults was observed and documented.

Bioassay

Details on bioassay were previously reported elsewhere (Whalon et al. 1993, Rahardja & Whalon 1995). Groups of ten to fifteen medium (0.6-0.8 g) second instars were exposed to treated potato leaves dipped in *B. thuringiensis* δ-endotoxin at 5-6 serial concentrations. Each bioassay was replicated three times at 25 ± 2 °C, 50-60% RH, and a photoperiod of 16:8 (L:D) h. The mortality was observed and documented 96 h after exposure, and the data subjected to probit analysis (Finney 1971).

Data Analysis

The method described here follows Tabashnik (1992). The realized heritability values are calculated as

$$h^2 = \frac{R}{S} \qquad (1)$$

where h^2 is the realized heritability, R or the response to selection is the difference in mean phenotype between the offspring of the selected parents and the parental generation before selection, and S or the selection differential is the average difference in mean phenotype between the selected parents and the parental generation before selection. R was estimated as

$$R = \frac{\log(LC_{50}F_n) - \log(LC_{50}F_i)}{n} \qquad (2)$$

where $LC_{50}F_n$ is the LC_{50} of offspring after n generations of selection and $LC_{50}F_i$ is the LC_{50} of the parental generation before n generations of selection. S was estimated as

$$S = i\,\sigma_p \qquad (3)$$

where i is the intensity of selection estimated using Appendix A of Falconer (1989) based on the percentage surviving selection. The average percentage of adults emerged from unselected colony was 30%. The proportion of mortality was adjusted using Abbot formula (Abbot 1952, Tabashnik 1992). The phenotypic standard deviation, σ_p, was estimated as

$$\sigma_p = \left(\frac{\text{slope}_i + \text{slope}_n}{2}\right)^{-1} \qquad (4)$$

where slope_i is slope of the initial probit regression line (Finney 1971) before selection, and slope_n is final slope after n generations of selection.

The responses to selection of each generation were also estimated by calculating the deviation of the log LC_{50} of resistant from the unselected colonies (Falconer 1973).

The correlations between the estimated and the cumulative selection deferential were plotted to determine the direction and the rate of the evolution of resistance.

The genotypic variance was observed following Tanaka & Noppun (1989). The standard deviations of the phenotype LC_{50} (1/slope) over all generations were calculated to determine the possibility of elimination of genetic variances as a consequence of inbreeding and selection.

The Coefficient of Inbreeding of both susceptible and resistance strains was estimated to determine the genetic variance of the strains. The inbreeding coefficient was calculated as a function of true population size (Wright 1942).

$$F_n = 1/(2N) + (1-1/(2N))F_{n-1} \qquad (5)$$

The backcross studies indicated that incompletely dominant gene(s) were involved in the resistance. The Colorado potato beetle strain was selected against CryIIIA endotoxin with a concentration of the toxin that produced over 95% mortality. If we assume that the selection causes elimination of the recessive individuals (SS), the proportion of S can be estimated from the number of adults that survived.

$$S = 1- (\% \text{ adult survived}/100)$$

In equilibrium populations with inbreeding consisting of two components: inbred individuals and random individuals (Li 1971). The average fitness of the inbred (W_I) and random (W_R) components is W, which is the sum of W_I and W_R where

$$W_I = 1 - sS, \text{ and } W_R = 1 - sS^2$$

and

$$W = F\, W_I + (1 - F)\, W_R$$

$$= F(1 - sS) + (1 - F)(1- sS^2)$$

Small s is the intensity of the selection for the recessive individuals and F is the coefficient of inbreeding.

The new frequency of the recessive (S') gene in the next generation (Wright 1942) is:

$$S' = 1/W \{ RS (1 - F) + S^2(1 - F)(1 - s) + SF(1 - s) \} \quad (6)$$

When s is 1, the equation (6) will be

$$S' = 1/W \{ RS (1 - F) \} \quad (7)$$

and the average fitness will be

$$W = F(1 - S) + (1 - F)(1- S^2) \quad (8)$$

RESULTS

The percentage of adults surviving after selection is reported in (Table II.1). There was a dramatic decrease in adult emergence at generations F_{10}. However, the resistance ratio (RR = LC_{50} resistant colony/LC_{50} susceptible colony) kept increasing every generation. The 29th generation of the resistant strain is 380 to 625 times more tolerant to *B. thuringiensis* CryIIIA δ-endotoxin than the susceptible strain (Rahardja & Whalon 1995).

The average responses to selection (R) for every generation were calculated. The values ranged from 0.01 (F_1) to 0.49 (F_4). The R values decreased after the fourth generation and reached values with very little fluctuation after the tenth generation. The deviations of the response (log LC_{50}) of the resistant colony from the unselected colony every generation were plotted against cumulative selection differentials, S (Figure II.1). The graph shows that in the first five generations, the resistance gains fluctuated

and then the values are very closely approximated to a straight line ($r=0.98$, slope=0.083, $F=210$, and $P<0.001$).

The estimate of realized heritability (h^2) of resistance to *B. thuringiensis* CryIIIA δ-endotoxin in Colorado potato beetle based at 29 generations was 0.17 (Table II.1). The highest value was 0.21 (F_4) and the lowest was 0.005 (F_1). After the tenth generation, the h^2 reached values which fluctuate very little (Figure II.2). The values fluctuated in a very narrow range (5%) over the last 12 generations. The estimated h^2 increased substantially at generation 29, in which the selection pressure was low (% adults emerged was 41).

Table II.1. Estimation of realized heritability (h^2) of resistance to *Bacillus thuringiensis* δ-endotoxin in Colorado potato beetle[a]

G	ADULT EMERGED (%)	LC_{50} mg/liter	SLOPE	R	S	h^2
G_1	0.90	1.63	1.80	0	1.50	0
G_2	0.40	0.68	1.10	-0.18	2.04	0
G_4	0.40	147	0.90	0.49	2.19	0.21
G_5	1.07	37	1.53	0.27	1.58	0.16
G_6	1.88	25	1.30	0.20	1.57	0.12
G_7	2.08	24	1.86	0.17	1.31	0.12
G_8	2.60	65	1.49	0.20	1.41	0.13
G_9	2.30	51	1.47	0.16	1.45	0.11
G_{10}	0.85	45	1.70	0.14	1.56	0.09
G_{12}	1.00	101	1.25	0.15	1.75	0.08
G_{13}	2.54	133	1.27	0.15	1.52	0.09
G_{15}	2.49	162	1.07	0.13	1.63	0.08
G_{16}	2.64	272	1.86	0.13	1.27	0.10
G_{17}	6.36	330	1.16	0.13	1.32	0.11
G_{21}	2.50	369	0.95	0.11	1.70	0.06
G_{29}	41	484	2.30	0.08	0.49	0.17

[a] G: Generation; Slope: the slope of the probit line; R or response to selection is the difference in mean phenotype between the offspring of the selected parents and the parental generation before selection; S or selection differential is the average difference in mean phenotype between the selected parents and the parental generation before selection.

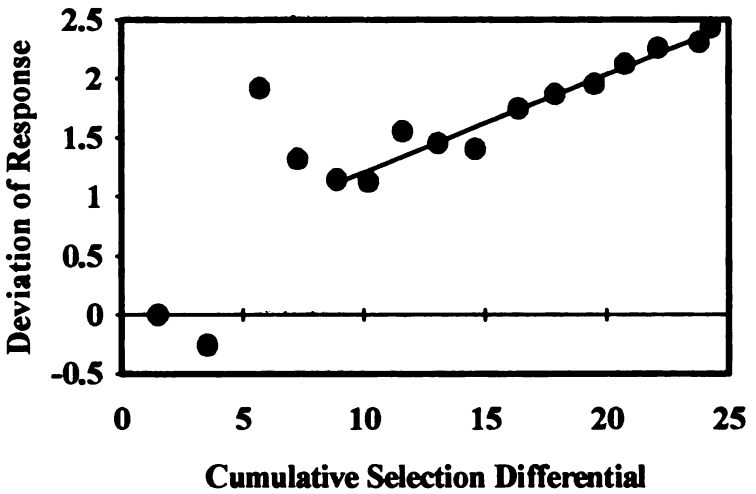

Figure II.1. The rate of change of response in Colorado potato beetle to selection by *Bacillus thuringiensis* δ-endotoxins CryIIIA. Each dot represents the gain (deviation of response of the resistant colony from the unselected colony) from the selection in each generation.

There was no significant correlation between the heritability estimates and the increment of resistance (r=0.27, P>0.05). However, the heritability estimates in the first 12 generations showed a relatively significant correlation with the increment of the resistance ratio (r=0.80, *P*=0.01).

Standard deviations of LC_{50}s over all generations (Figure II.2) were not significantly different between selected and unselected colonies. (t=-1.84, df=25, P=0.08). This indicates that the standard deviations in both strains are of the same magnitude.

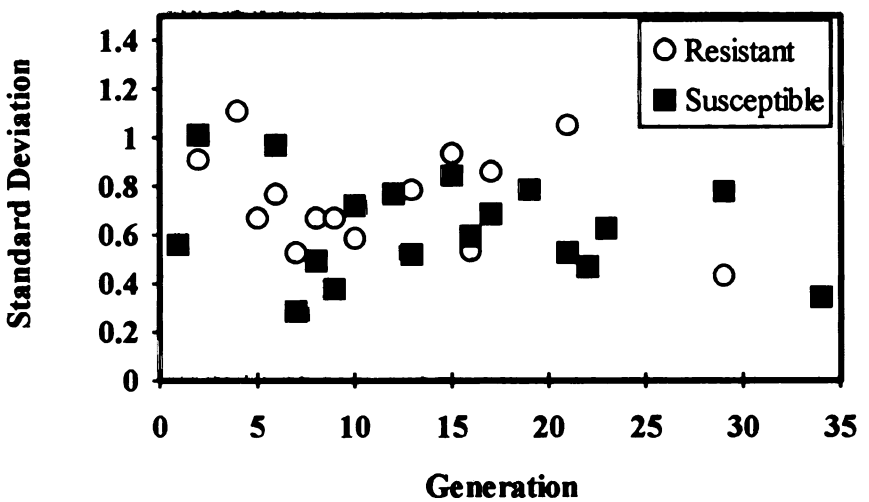

Figure II.2. Standard deviation (1/slope) of LC_{50} values of the *Bacillus thuringiensis* δ–endotoxin selected and unselected strains of Colorado potato beetle.

Phenotypic standard deviations are not significantly correlated with the number of generations (Table II.2). This indicates that there were no dramatic changes in phenotypic variances over many generations in both the selected and unselected populations. However, the variance does appear to decrease slowly through the experiment in both

strains. The negative slopes of the regression lines between the number of generations and standard deviations of both colonies indicate that the genetic variance decrease over generations. In general, genetic variance decreases as a result of directional selection (Falconer 1989).

Table II.2. Correlation regression analysis between standard deviation of LC_{50}s and generation number

STRAIN	N	r	slope	*P*
Resistance	16	0.11	-0.003	0.69
Susceptible	18	0.17	-0.004	0.50

The principle effect of inbreeding in a population is to increase the frequency of homozygous genotypes. Thus, inbreeding measures the reduction of the frequency of the heterozygous individuals.

Fixation is the consequence of inbreeding. Fixation occurs in a line when the line becomes homozygous for the same allele at a particular locus. F will be equal to one when the same allele is fixed in a population. The frequency of the homozygous individuals is 1, too.

CryIIIA endotoxin resistance selection in Colorado potato beetle was started in six Michigan field sites representing different insecticide use histories. The density of Colorado potato beetle larvae was estimated to be between 2 - 6 million. After 2 years of

field selection, approximately 25,000 surviving beetles were collected and transported to the laboratory. The field selected beetles were maintained and randomly mated for

Table II.3. Various values of Coefficient of Inbreeding (F)

Generation	Number larvae selected	% Survived	N_A	F_{ACTUAL}	F_{TN} N = 25,000	F_{TS} N = 25,000	F_{TSA} N=N_A	$F_{SUSCEPTIBLE}$ N = 250
0	2E+06		25000.00	0.00002	2.5E-07	2.03E-05	0.002228	2.5E-07
1	34933	0.9	314.39	0.00161	2.02E-05	4.15E-05	0.003909	0.002
2	7909	0.4	31.63	0.01739	4.02E-05	6.23E-05	0.020254	0.003992
4	14125	0.4	56.50	0.026085	8.02E-05	0.000103	0.031698	0.007952
5	18186	1.07	194.59	0.028588	0.0001	0.000124	0.033611	0.009921
6	13664	1.88	256.88	0.030479	0.00012	0.000144	0.035181	0.011881
7	14974	2.08	311.45	0.032035	0.00014	0.000164	0.037189	0.013833
8	9329	2.6	242.55	0.03403	0.00016	0.000184	0.039782	0.015778
9	8137	2.3	187.15	0.036611	0.00018	0.000204	0.049386	0.017715
10	5921	0.85	50.32	0.046182	0.0002	0.000224	0.072888	0.019644
12	2034	1	20.34	0.069629	0.00024	0.000265	0.086124	0.023479
13	4210	2.54	106.93	0.073979	0.00026	0.000285	0.093911	0.025385
15	2096	2.49	52.19	0.082851	0.00030	0.000325	0.119767	0.029175
16	2231	2.64	58.89	0.090637	0.00032	0.000345	0.120844	0.031058
17	912	6.36	58.00	0.098475	0.00034	0.000365	0.12525	0.032934
21	1000	2.5	25	0.116506	0.00042	0.000445	0.142648	0.040362
29	1000	41	410	0.117583	0.00058	0.000605	0.176388	0.054866

N_A = Actual number of beetles survived after selection; F_{ACTUAL} = F calculated based on N_A; F_{TN} = Hypothetical F calculated with no selection; F_{TS} = Hypothetical F calculated with selection; F_{TSA} = Hypothetical F calculated with selection and based on N_A; $F_{SUSCEPTIBLE}$ = F calculated based on average number per generation used for laboratory culture

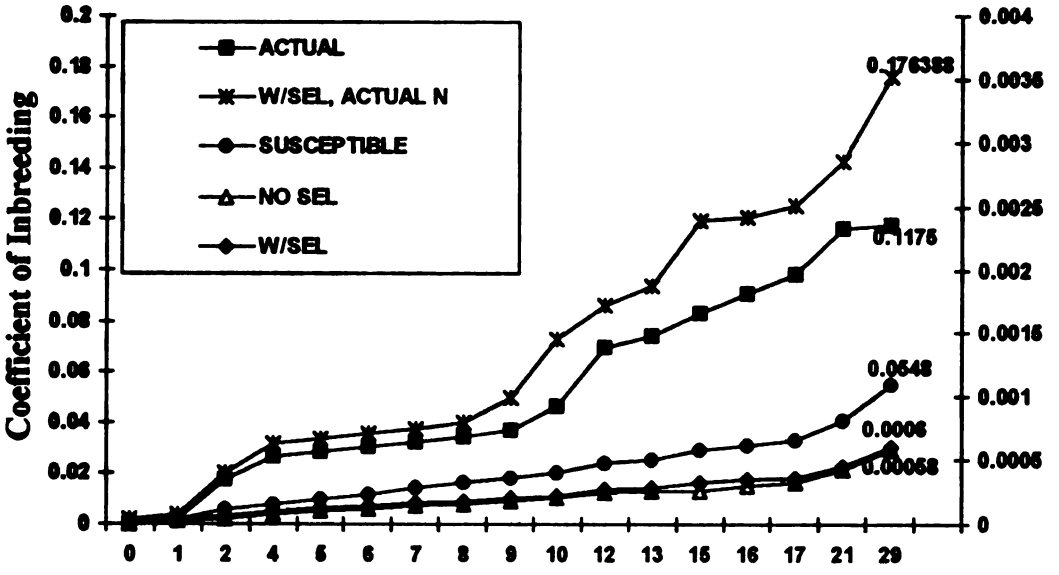

Figure II. 3. Coefficient of Inbreeding (F) values of Colorado Potato Beetle strains after 29 generations of selection

continuous selection in our laboratory. The table below shows the number larval survival and adult emergence for each generation of selection.

Where F is the coefficient of inbreeding; n represents the generation number and N represents the population size. Since the initial population for selection was collected from the field with an estimated population size of 2 - 6 million, we can assumed that the F for the initial generation would be at the most 1/(2*2,000,000) or 0.00000025.

F_{ACTUAL} was calculated based on the actual number of adults emerging after selection in each generation. This value represents the coefficient of inbreeding as a function of true population size with selection. Thus, the population size being selected plays a role in delaying the fixation process in both strains (resistant and susceptible). The F value in the last generation (G_{29}) was 0.12 (Table II.3 and Figure II.3). This value

indicates that fixation (F = 1) has not been reached, and only 12% of the population was homozygous.

F_{TN} is the F value of a population without selection with a hypothetical constant population size of 25,000 throughout 29 generations (see Table at pg 4 Figure at pg 5). The value was calculated using formula (1). Under this assumption, after 100 generations the F value would still be very small (=0.001). When the population size is large, the fixation process of the population is slow.

F_{TS} is the F value of the population where a selection is in effect and the population size being selected is constant (=25,000). The new proportion of RR and RS genotypes after selection in every generation were calculated with the assumption that s is 1 (i.e. elimination of SS) and the initial frequency of S is the frequency in the first generation of selection (=0.99549). The population size after selection would be proportional to the sum of the frequencies of RR and RS. Since fitness (W) of the population was also calculated every generation, this model yielded a very small F_{TS} value (=0.0006) at generation 29. When the actual population size every generation was used, the calculated F_{TSA} after generation 29 was 0.17 (Table II.3 and Figure II.3). It is clear here that the population size determines the rate of change in F; a higher value of F can be reached in a shorter time in a population with a smaller size.

As expected, the F_{TSA} value at generation 29 is higher than the F_{ACTUAL}. Selection may cause reduction in genetic variance. The loss of genetic variance should lead to reduced phenotypic variance. The phenotypic variance, however, is seldom found to decline as expected; often it increases. The possible reason for the phenotypic variance not decreasing may be that mutations contribute to maintaining the genetic variance. Since the model to calculate F_{TSA} excludes the possibility of mutation as a source of heterozygosis or hereditary variation of a population (Li 1955, Falconer 1989), it underestimates potential heterozygosity.

The coefficient of inbreeding was calculated for the susceptible strain ($F_{SUSCEPTIBLE}$) by using equation (5). The average population size was approximately 250 per generation.

After 29 generations, the $F_{SUSCEPTIBLE}$ value was 0.055. The lower $F_{SUSCEPTIBLE}$ value in comparison with the F_{ACTUAL} was expected since the selection againsts deleterious recessive allele (in this case: susceptible allele which is recessive) will not delay the fixation of the more favorable allele (Falconer 1989).

After 29 generations of selection, the phenotypic variance of both strains was still higher than expected. From the value of the Coefficient of Inbreeding calculated above, it is clear that the genetic variation, thus phenotypic variation, has not decreased dramatically. Thus the selection or inbreeding processes have not led to the fixation of the gene(s).

DISCUSSION

Adult emergence in the selected colony at generation G_{10} decreased dramatically from 2.3 % to 0.85 %, while no such decreased was observed in the unselected colony. However, there was no significant different in the resistance level and heritability values. The results indicate that while gaining the ability to tolerate the δ-endotoxin, the selected colony suffers a disadvantage relative to the susceptible colony. This phenomenon is in agreement with the theory that selection may result in depression of the vigor of the population (i.e. reduction of fertility)
on which the selection acts (Dobzhansky 1990). The reduction in adult emergence may be an indication of cost of resistance in Colorado potato beetle population.

Heritability values presented here were estimated based on the additive variance and phenotypic variance. A numerical value of realized heritability of 1 means that the offspring exactly resemble the selected parents; on the other hand, heritability of zero means no correlation between the parents and their progeny. Heritabilities in many studies

on insects generally range between those two extremes (see Falconer 1989, Tanaka & Nopun 1989, Firko & Hates 1991, Tabashnik et al. 1991, 1992, Aspi & Hoikkala 1993, and Omer et al. 1993). Table II.3 shows the estimates of the heritability of various phenotypes in various animal species (after Falconer 1989). There is some connection between the magnitude of the heritability and the nature of the phenotype. The phenotypes with the lowest heritabilities are those most closely related with reproductive fitness, while phenotypes with the highest heritabilities are those that might be considered to be remotely related with natural fitness (Falconer 1989).

Heritability estimates reported here were done under uniform laboratory conditions; thus our estimations resulted mainly from the phenotypic variability in our populations. After three successive selections the heritability estimate was 0.21. The peak value was accompanied by a dramatic increment in the resistance ratio (Rahardja & Whalon 1995) at generation G_4 (Table II.1). The decrease of heritability estimates was not followed by the decline of the resistance ratio (r=0.27, P=0.30). Furthermore, the heritability values do not correspond with the major shift in resistance gain that occured between the G_3-G_4, G_7-G_8, G_{11}-G_{12} (Whalon et al. 1993) and G_{16}-G_{17}(Table II.1). The heritability estimates were calculated based on the level of LC_{50} regression slope before and after selection and mortality caused by selection. With the assumption that the heritability value and the slope are constant, the effect of selection pressure toward the selection gain will increase. For example, if the $\sigma p = 0.64$ (based on the reciprocal of the mean slope from generations 1-21), $h^2 = 0.097$ (the mean of h^2 from generations 1 - 21), and 50% of the population is selected every generation (p =50%, $i = 0.798$), the response to selection (R) is 0.046. If 90% of the population are killed ($i = 1.755$), the R value increases to 0.10.

Table II.3. Approximate values of the heritability of various characters in various animal species. The estimates are rounded to the nearest 5 per cent; their standard errors range from about 2 per cent to about 10 per cent (Falconer 1989).

	h^2 (%)
Man	
Stature	65
Serum immunoglobulin (IgG) level	45
Cattle	
Body weight (adult)	65
Butterfat, %	40
Milk-yield	35
Pigs	
Back-fat thickness	70
Efficiency of food conversion	50
Weight gain per day	40
Litter size	5
Poultry	
Body weight (at 32 weeks)	55
Egg weight (at 32 weeks)	50
Egg production (to 72 weeks)	10
Mice	
Tail length (at 6 weeks)	40
Body weight (at 6 weeks)	35
Litter size (1st litters)	20
Drosophila melanogaster	
Abdominal bristle number	50
Body size	40
Ovary size	30
Egg production	20

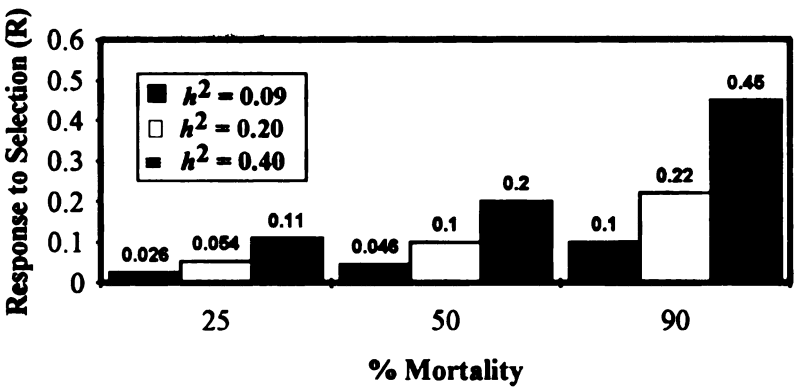

Figure II.4. Effects of heritability and percentage mortality on the average response per generation of Colorado potato beetle to selection with *Bacillus thuringiensis* δ-endotoxin

The graph of Figure II.3 shows that when the selection pressure (% mortality) increases, the reponse to selection will also increase. The h^2 value also contributes to the magnitute of the response to selection. The higher the heritability value, the higher the response, therefore, the quicker the development of resistance. Similar projections have been made by Tabashnik (1992) and Omer et al. (1993). They concluded that resistance to toxic agents develops faster as h^2 and selection intensity increase.

The mean estimated heritability value after 29 generations is relatively low (h^2 = 0.10). This value is not suprisingly low compared with other heritabilities of other insect pests with the exception of *Plodia interpunctella* (Table II.4). The heritability values of resistance to *B. thuringiensis* δ-endotoxin in several insect pests range from 0.05 to 0.17. The heritability estimates for *P. interpunctella*, however, range from 0.29 to 0.35. The relatively high heritability estimates obtained for *B. thuringiensis* δ-endotoxin are consistent with rapid development of resistance of Indian meal moth (McGaughey 1985) population in treated grain bins.

Resistance development rates can be projected by rearranging Equation (1) (Tabashnik 1992). The projected response to selection increases as heritability value and selection pressure or intensity increase (Tabashnik 1992, Omer et al. 1993). In principle, therefore, the number of generations required for ten-fold increase in LC_{50} decreases as heritability value and the proportion of the population killed by the insecticide increase. In this study, we projected the rates of resistance development based on the heritability value of generation G_4 (h^2 =0.21). The expected number of generations required for ten-fold increase in LC_{50} ($G=R^{-1}$) was 5. The expected and observed number of generations were in agreement. However, further projections for the number of generations required for 100 and 200-fold increase in LC_{50} ($G=R^{-2}$ and $G=R^{-2.301}$) were not in agreement with the observed values. As Dobzhansky (1970) observed, the selection gains can be predicted fairly well for several initial selections if the heritability of the trait is known.

Falconer (1989) stated that if there is a response to selection, the frequency of the gene conferring resistance must change. Thus the heritability values, which depend on gene frequency, will also change. This change is not likely to be apparent for considerable time because gene frequency changes are small unless only a few loci involved. In the early generations, selection will reduce the variance and the heritability (Falconer 1989). The expected changes in heritability are not large. Our data seems to follow this proposal. After reaching the peak at generation four, the heritability estimates of the following generations drop off and reach a stable value with very little fluctuation. The values range from 0.06 to 0.12.

Crow (1957) suggested that epistatic interaction can evolve under close inbreeding. As we mentioned in another report (Rahardja & Whalon 1995) this statement suggests a simple relationship between genes in which each might make a contribution to the resistance development in the selection process of Colorado potato beetle. As a consequence, the frequency of genes conferring resistance (and the h^2) changes very little over generations, but the population keeps gaining the ability to overcome the selection. Furthermore, when a particular gene accounts for only a small portion of the total phenotypic variance, the gene frequency does not change very rapidly (Falconer 1989).

The responses to selection of the resistant strain (deviation of response of the resistant colony from the unselected colony) over 29 generations were plotted against cumulative selection differential for the resistant strain (Figure II.1). The responses fluctuated erratically at the first five generations before reaching a steady rate of increase. The slope of the regression line was relatively small indicating that the rate of the selection gain was slow. The graph does not suggest that the selection limits were reached after 29 generations of selection. This is in agreement with the changes in genotypic variances that have not yet reached a plateau.

Table II.5. Estimates of realized heritability (h^2) of insecticide resistance

Species Insecticide	h^2	Reference
Culex quinquefasciatus		
Temephos	0.4	Ferrari et al. (1982)
Permethrin	0.39	Ferrari et al. (1982)
Popoxur	0.25	Ferrari et al. (1982)
Heliothis virescens		
Cypermethrin	0.85	Firko & Hayes (1991)
Bacillus thuringiensis subsp. *kurstaki*	0.17	Stone et al. (1989)[a]
Plutella xylostella		
Penthoate	0.42	Tanaka & Nopun (1989)
Fenvalerate	0.20	Tabashnik & Cushing (1989)
B. thuringiensis subsp. *kurstaki*	0.16	Tabashnik et al. (1991)[a,b]
B. thuringiensis subsp. *thuringiensis*	0.05	Devriendt & Martouret (1976)[a]
Sitophilus oryzae		
Pirimiphos-methyl	0.47	Holloway (1986)
Plodia interpunctella		
B. thuringiensis subsp. *kurstaki*	0.35	McGaughey & Beeman (1988)[a]
B. thuringiensis subsp. *aizawai*	0.32	McGaughey & Johnson (1992)[a,b]
B. thuringiensis subsp. *entomocidus*	0.29	McGaughey & Johnson (1992)[a]
Trialeurodes vaporariorum		
Dicrotophos	0.40	Omer et al. (1993)

In general, the expected response to selection is measured by the reduction of phenotypic variance. The changes in variance presumably are due to the changes in gene frequency (Falconer 1989) yet we did not observe a significant decline in our resistant strain. The laboratory selection process initiated with field population might be the reason why the susceptible and resistance strains still preserve a relatively large genetic pool. Phenotypic variance (σ_P^2) can be partitioned into genotypic variance and environmental variance. The genotypic variance (σ_G^2) is due to dominance, recessiveness, over-dominance (σ_D^2), to the epistatic interaction of genes at different loci (σ_I^2), or may be entirely due to the differences between genotypes in the population (Dobzhansky 1970). The lack of significant systematic changes in phenotypic (LC_{50}) variances in the last 14 generations (generations 16-29) may have occurred as a result of the existence of the partial dominant gene(s) conferring the resistance toward δ-endotoxin in Colorado potato beetle (Rahardja & Whalon 1995).

However, the variance of LC_{50} values seemed to decrease in both selected and unselected colonies. The effect of inbreeding in both laboratory strains and directional selection from our rearing system may start affecting the phenotypic variance and may have contribute to this reduction. In the absence of mutation, the response to selection cannot be expected to continue indefenitely. Eventually, the genes segregating in a population will be brought to fixation by selection (Falconer 1989). The response will slowly diminish and finally cease. The population reaches a plateau or selection limit. At the limit, all favorable alleles at all loci will be homozygous.

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CHAPTER III

Random Amplified Polymorphic DNA Markers For Genes Conveying Resistance To *Bacillus thuringiensis* subspecies *tenebrionis* CryIIIA δ-endotoxin In Colorado Potato Beetle (Coleoptera: Chrysomelidae)

INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) is one of the most destructive pests on potato and is resistant to a wide range of insecticides, including chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids (Gautheir et al. 1981, Georghiou 1986, Grafius 1986, Ioannidis et al. 1991, 1992). Colorado potato beetle resistance to synthetic organic insecticides in the eastern and midwestern United State has contributed to a marked increase in the use of *Bacillus thuringiensis* CryIIIA δ-endotoxin.

In 1987 field populations of Colorado potato beetle were treated with the CryIIIA δ-endotoxin of *B. thuringiensis* (Whalon et al. 1993) and the survivors underwent further laboratory selection which resulted in over 200 fold resistance to the δ-endotoxin (Rahardja & Whalon 1995). The resistance is autosomaly inherited and there is no maternal influence (Rahardja & Whalon 1995).

The development of the *B. thuringiensis* CryIIIA δ-endotoxin resistance in the Colorado potato beetle in laboratory (Whalon et al. 1993) raises concerns that broad scale resistance to *B. thuringiensis* CryIIIA δ-endotoxin may occur in field populations. Obviously, there is a need to develop a program for managing resistance to *B. thuringiensis* CryIIIA δ-endotoxin in Colorado potato beetles.

Resistance monitoring is an important feature of any resistance management program. An effective tool to monitor resistant phenotype in field populations can supply valuable information for developing strategies for managing resistance. Ideally, a monitoring tool should detect resistance as it begins to develop in the field. Thus providing initial warning that could be useful for predicting the number of generations before resistance develops, and which will allow time to take the necessary steps to manage resistance. Roush and Miller (1986) concluded that the use of LD_{50}s, the slope of probit regression line and the LD_{95}s seems to be adequate for resistance detection

when the resistance has reached high levels. However, lethal dosages are very inefficient for early detection of an incipient resistance outbreak. A program to detect resistance before a pesticide fails in the field requires a high level of accuracy in estimating the frequency of alleles conveying resistance to the pesticide.

Genetic analysis can be simplified by the availability of DNA markers showing multiallelic variation which correspond or cosegregate with target traits. Genetic markers can be developed from DNA polymorphism based on the amplification of random DNA segments with a single oligonucleotide primer. This polymorphism, detected as DNA fragments which are amplified from one parent but not from the other, are inherited in a Mendelian fashion and can be used to construct genetic maps (William *et al.* 1990). This approach is called Random Amplified Polymorphic DNA (RAPD) which makes use of the Polymerase Chain Reaction (PCR) technique. The RAPD system is currently used in many different organisms to construct genetic maps, mark important genes, and to study population genetic structure (Waugh & Powell 1992, Chapco et al. 1992, Black et al. 1992).

In this study, we attempted to implement the PCR-RAPD technique to develop DNA markers for gene(s) conferring resistance to *B. thuringiensis* CryIIIA δ-endotoxin in Colorado potato beetles. When amplified polymorphic DNA fragments are detected, further analysis should then be done to determine whether the polymorphism can be used as a diagnostic tool for the resisitanct genes. After validating RAPD markers on the resistant population, the next logical step is to go to the field to correlate the results of bioassays with fingerprinting using RAPD markers. This will determine the usefulness of these probes by evaluating the possibility of detecting resistant individuals in the populations. We proposed that genetic markers that detect genes responsible for Colorado potato beetle resistance to *B. thuringiensis* CryIIIA δ-endotoxin will provide early detection of resistance in the field.

The potential for development of field resistance to pesticides depends on genetic and biological factors of the pests combined with environmental and operational characteristics of an agroecosystem. The selection gain toward resistance in each generation can be predicted from the heritability values of the selected phenotype (Dobzhansky 1970). The estimation of the realized heritability values from the laboratory selected Colorado potato beetle colony was reported in a previous report (Chapter II). When the RAPD primers recognize positive individuals in a field population, it provides the opportunity to estimate the realized heritability of that population which is a way of assessing resistance potential or risk (Tabashnik 1992). Successful completion of this process can confirm the usefulness of the RAPD primers and the potential development of resistance in the sample populations. This study would determine a genetic basis for *B. thuringiensis* δ-endotoxin resistance, and an ideal diagnostic tool for its detection.

The utility of DNA-based diagnostic markers is determined to a large extent by the technology that reveals DNA-based polymorphism. Currently, the technology for amplification of discrete loci with single, arbitrary, random sequence of oligonucleotide primers is popular because of its simplicity and ease of use in a modestly equipped laboratory. These RAPD assays detect nucleotides sequence polymorphism in a DNA amplification-based assay using only a single random primer. Several applications of the RAPD technique have been developed. Each of these technique exploits the efficiency of detection of the polymorphism. The application of this technique is attractive because it requires only small amounts of DNA as a template and also can be performed in several hours. RAPD analyses of single insect have been the only viable approach for genetic mapping and population genetic studies in very small insects (Landry et al. 1993). These significant advantages provide an excellent approach for field detection of resistance evolution. Furthermore, developing genetic markers for genes conferring resistance to *B. thuringiensis* δ-endotoxin in Colorado potato beetles will open further understanding of resistance inheritance.

The objectives of this study were: 1) to evaluate the RAPD system for segregation of *B. thuringiensis* δ-endotoxin resistant and susceptible alleles in controlled crosses of CPB strains, 2) to validate the use of RAPD markers as a diagnostic probe for *B. thuringiensis* δ-endotoxin resistance in field populations.

MATERIALS AND METHODS

A. Detection of Colorado Potato Beetle Resistance to *Bacillus thuringiensis* subspecies *tenebrionis* δ-endotoxin: Linkage Analysis of RAPD Markers

DNA Isolation

Genomic DNA from single Colorado potato beetle third-fourth instars or adult (susceptible and Bt-R strains) was extracted following Bender et al. (1983) with some modifications. The wings of beetles were removed prior to homogenization. An individual was ground in a microtube using a disposable pestle in 250 µl of lysis buffer (0.1 M NaCl, 0.1 M Tris-HCl pH 9.0, 0.2 M sucrose, 0.05 M EDTA and 0.5% sodium didoxyl sulfate). The mixture was incubated for 30 min at 65°C. After incubation, ammonium acetate (7 M, pH 7.4) was added immediately to give final concentration of 2.5 M. The mixture was incubated on ice for at least 30 min before the DNA was separated from other macromolecule by centrifugation for 15 min at maximum speed (14,000 rpm). The supernatant was collected and transferred to fresh 1.5 ml tube. The DNA was precipitated by adding 1 ml of ice-chilled absolute ethanol and incubate at -20°C for at least 30 min. Alternatively, the mixture was incubated at room temperature for 5 min but no longer than 10 min. The precipitant was collected by spinning the mixture at 14,000g for 15 min. The ethanol supernatant was discarded and the pellet was washed by adding ice chilled 70% ethanol, vortexed and spun at 14,000g for 5 min. The pellet was

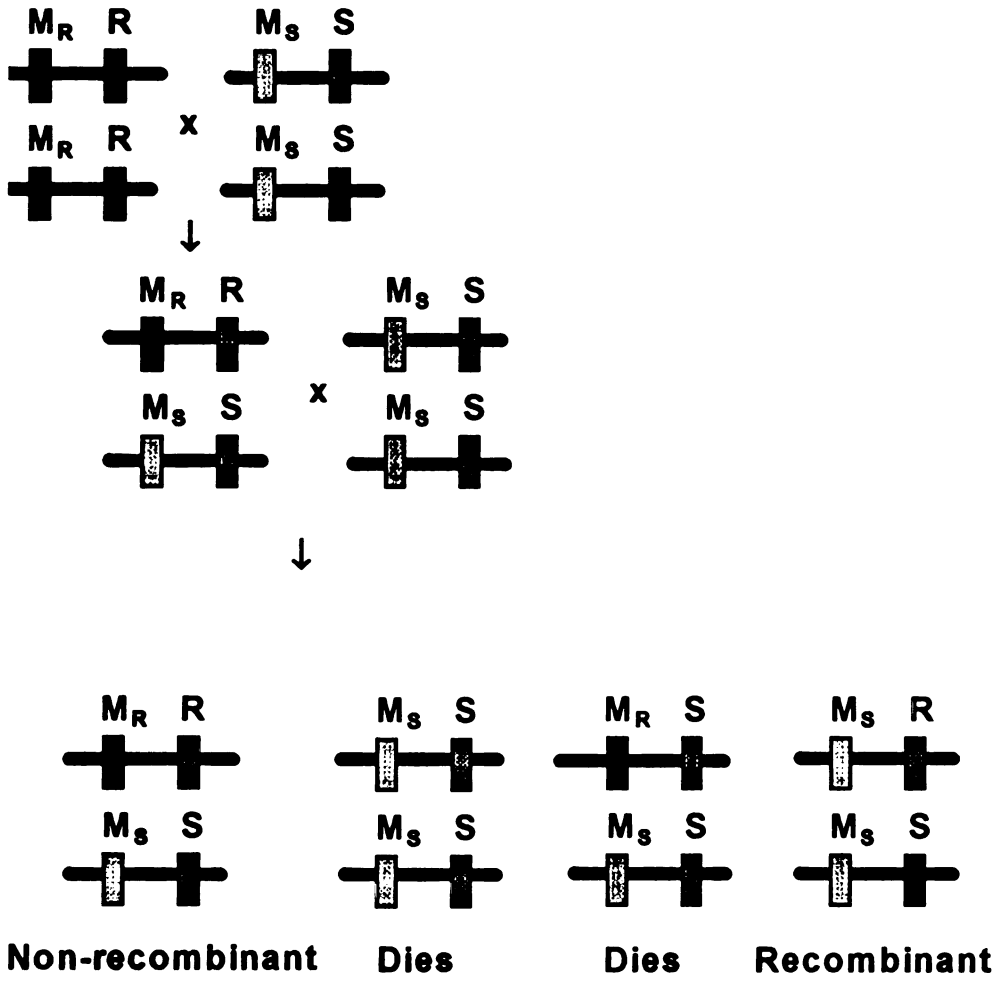
dried in a vacuum dessicator and redissolved with 100 µl TE buffer (0.01 M Tris-HCl pH 8.0, 0.001 M EDTA).

DNA Amplification

DNA amplifications were performed in a volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 100 mM each of dATP, dCTP, dGTP, dTTP, 5 pica mole of primer, 25 ng of DNA template, and 1.5 U of Taq DNA polymerase. More than 200 ten-base primers were analyzed (Appendix III.1). Amplifications were performed on a thermocycler programmed for 45 cycles as follows: 1 minute at 92°C, 1 minute at 35 °C, slope up to 72 °C for 5 minute, and 2 minutes at 72 °C. Amplifications products were analyzed by electrophoresis in 1.4% agarose gels and were run in a model DNA Sub Cell gel box (Biorad) at 4-8 V cm^{-1} for 4-6 hours. At the end of electrophoresis, gels were stained with ethidium bromide (5 ng/ml) for 30 min and photographed under UV light using a DS34 camera (Polaroid) and Polaroid type 667 film.

Linkage Analysis

Appropriate inbreeding, reciprocal crosses between susceptible and resistance strains (yield F_1 progeny), backcrosses between F_1 progeny and the homologous susceptible parents were carried out (Rahardja & Whalon 1995) for linkage analysis. The DNA of the parent and offspring of each cross was extracted and subjected to RAPD analysis. Before being extracted, the second instar of the offspring were screened with a discriminating dose of δ-endotoxin (60 mg CryIIA δ-endotoxin per liter of water). Surviving fourth instars were used for the linkage analysis (see flow chart in Figure III.1).



MR = Marker for resistant gene

MS = Marker for susceptible gene

R = resistant gene

S = susceptible gene

Figure III.1 The genotype of the progeny of the backcross between F_1 (from reciprocal cross) and Susceptible strain.

The recombination fraction between the amplified DNA fragment and the gene for resistance depends on the position of genetic marker to the resistant gene(s) on the chromosome(s). If the marker and resistant gene are on different chromosomes, the expected recombination fraction is 0.5. If the genetic marker and the gene are on the same chromosome, the recombination fraction is determined by their separation distance. When the recombination fraction is less then 0.5, the genetic marker and the gene must be on the same chromosome. The closer they are on the chromosome, the less likely they will undergo crossing over, while the farther apart they are the more likely a cross over will occur. The recombination fraction (r) was determined as:

$r = 1-(p/n)$ (Lewin 1990)

where **p** is the number of survivors from back crosses that carried the genetic marker(s) and **n** is the total number of progeny. The offspring used in these experiments were the survivors from discriminating dose exposure. The null hypothesis underliying this process is that the proportion of recombinants between resistant locus and the marker locus is equivalent to the number of non-recombinants.

B. Detection of Colorado Potato Beetle Resistance to *Bacillus thuringiensis* var *tenebrionis* δ-endotoxin: Validation and Field detection of RAPD Markers

Field Evaluation

Whenever amplified polymorphic DNA fragments are detected, further analysis should then be done to determine whether the polymorphism can be used as a diagnostic tool for the targeted trait. The DNA primers which were previously shown to be diagnostic for CryIIIA δ-endotoxin resistance in controlled crosses were used to evaluate field populations. Colorado potato beetles at any developmental stages found in the field were collected from nine different field populations during the 1993 Summer (Table III.1), brought to the laboratory and stored in -70 °C before being tested. Each sites' rotation

pattern and pesticide application history (three previous years) was documented (Table III.1). A number of second instar from each population were submitted to a discriminating dose (Rahardja & Whalon 1995) to determine their susceptibility to *B. thuringiensis* CryIIIA δ-endotoxin.

DNA Extraction From Field Samples

Genomic DNA from field collected Colorado potato beetles was extracted as above and amplified with the diagnostic primers previously identified. A sequential sampling scheme was employed to reduce the amount of expensive Taq polymerase enzyme necessary to classify each sample. Initially, DNA was extracted from groups of 50 individuals from the same site. After RAPD analysis, samples which yield positive results with the primer were reanalyzed in groups of 10, and then individually. Since ng quantities of DNA is used, further verification of positive individuals could be done by taking more DNA samples of the same alive or frozen field collected Colorado potato beetles.

RESULTS

Development of DNA Markers

Initially, over 200 ten-base oliginucliotide primers were evaluated for DNA markers for *B. thuringiensis* δ-endotoxin resistance gene/s in Colorado potato beetle (Appendix III.1). For each primer, twenty beetles (ten for susceptible and 10 for resistant beetles) were used for the screening. Any primers that showed polymorphism between susceptible and resistant strains were further tested. Among those, 12 primers were tested against 50-200 beetles individually from the resistance and susceptible colonies. Two of these 12 primers, R-14 and R-17, produced diagnostic fragments for the laboratory colonies (Figure III.2 and Figure III.3).

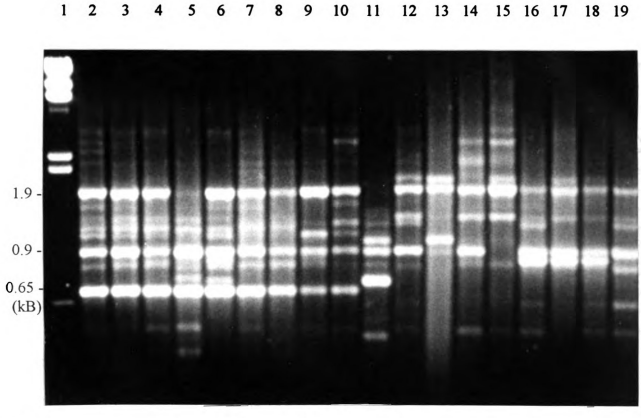


Figure III.2. PCR products of Colorado Potato Beetles amplified by R-14. Each lane represents one individual. Lane 1 is lambda HindIII marker, lane 2-10 are resistant individuals, and lane 11-19 are susceptible individuals

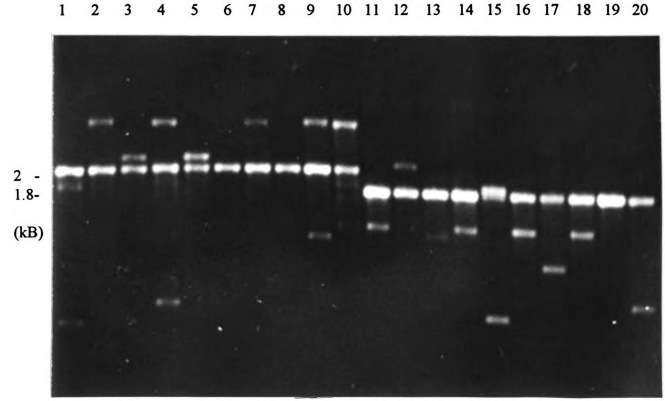


Figure III.3. PCR products of Colorado Potato Beetles amplified by R-17. Each lane represents one individual. Lane 1-10 are susceptible individuals, and lane 11-20 are resistant individuals.

Table III.1. History of Colorado potato beetle field populations tested with the RAPD primer R-17 for *Bacillus thuringiensis* CryIIIA δ-endotoxin resistance.

Population	Planting pattern[a]	County	Individual test[b] +/total[c]
Clio 1	P - P - P (-)	Genesse	0/150
Clio 2	P - P - P (-)	Genesse	0/200
Imlay 1	P - P - P (-)	Lapeer	0/50
Imlay 2	P - P - P (-)	Lapeer	0/150
Imlay 3	P - P - P (-)	Lapeer	0/50
Lansing	P - P - P (-)	Ingham	0/50
SWMREC	T - T - T (-)	Van Buren	7/250
BQ	T - T - T (-)	Van Buren	2/200
Lakeview	P - P - P (-)	Montcalm	0/50

[a] Rotation pattern in 1991, 1992 and 1993. P: potato, T: tomato
(-): No *B. thuringiensis* CryIIIA δ-endotoxin spray experience

[b] N=number of samples tested in groups of fifty larva

[c] +/total= number of individuals exhibited diagnostic marker/total number individual tested

Linkage Analysis

The RAPD linkage analysis was based on six crosses resulting from pairwise mating between selected F_1 reciprocal cross progeny and individuals from the susceptible strain. The resistance level in the progeny from these backcrosses was determined before DNA extraction. Each individuals from backcrosses were scored with respect to their parental RAPD as either homozygous for the maternal or paternal type or as a heterozygote.

The R-14 primer (ACAGGTGCTG) yielded 16 different amplified DNA fragments ranging from 300 to 3,000 base pairs (bp) in size (Figure III.2). DNA fragments of 900 and 1,900 bp were amplified in most individuals, both susceptible and resistant. In the resistant individuals, there was another fragment (650 bp) that was amplified in all the samples. The R-17 primer (CCGTACGTAG) amplified 10 different DNA fragments ranging from 900 to 3,200 bp in size. Fragment 2,020 bp was amplified only in susceptible individuals. This fragment was not present in the resistant colony, but a 1,800 bp fragment was amplified instead (Figure III.3 and III.4). However, in the heterozygous F_1 individuals, the polymorphism resembled the resistant parents. The 2,020 bp fragment was not observed in heterozygous F_1 individuals.

Of the two diagnostic fragments (650 bp and 1,800 bp) only the 1,800 bp showed linkage to the gene conferring resistance to CryIIIA δ-endotoxin. The test for linkage showed no significant deviation between the number of individuals that showed the 650 bp resistant marker (non-recombinant individuals, NR) and the number of individuals that did not show the 650 bp resistant marker (recombinant individuals, R) (Table III.2). The result indicates that the 650 bp marker was not linked to the gene conferring resistance. However, significant deviation ($P<0.01$, $df = 1$) from the 1NR:1R ratio for 1,800 bp marker was observed in the backcross progenies. The 1800 bp fragment lies significantly linked to the gene, and its presence can be used to detect the frequency of the gene in field population. Although the marker is linked, the distance in terms of DNA base pairs can be substantially far apart. However, the fragments can be used as a starting point to isolate the gene itself.

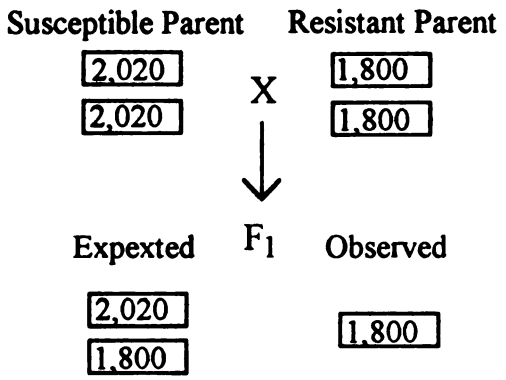


Figure III.4. Diagram of the expected and observed phenotype of F_1 progenies from reciprocal cross between susceptible and resistant parents. 2,020 is the RAPD-PCR fragment diagnostic for susceptible phenotype and 1,800 is the RAPD-PCR fragment diagnostic for resistant phenotype generated by R-17 primer.

Table III.2. Chi-square analysis of RAPD-PCR products of back cross progeny.

Primers	N	NR	R	χ^2
R-14	122	67	55	1.18
R-17	122	95	27	37.90**

N : The total number of individual CPB analyzed. The second instar of the progeny from back cross of F_1 X Susceptible parent were treated with 60 mg/L of CryIIIA δ-endotoxin. Only the fourth instar survivors were used for the study; NR : the number of individuals showed the resistant marker (non-recombinant individuals), R : the number of individuals did not show the resistant marker (recombinant individuals). Null hypothesis NR:R is 1:1.
**χ^2 = 6.63 with d.f = 1 at P = 0.01

Validation and Field Detection of RAPD Markers

The primer (R-17) gave the fragment that is linked to the resistant phenotype in laboratory Colorado potato beetle may have a strong potential to be used as a field diagnostic tool. Populations of Colorado potato beetle from nine different locations were used to test the usefulness of the primers for field detection (Figure III.5). The susceptibility was checked by exposing 30-90 second instars to 30 mg/liter of CryIIIA δ-endotoxin.

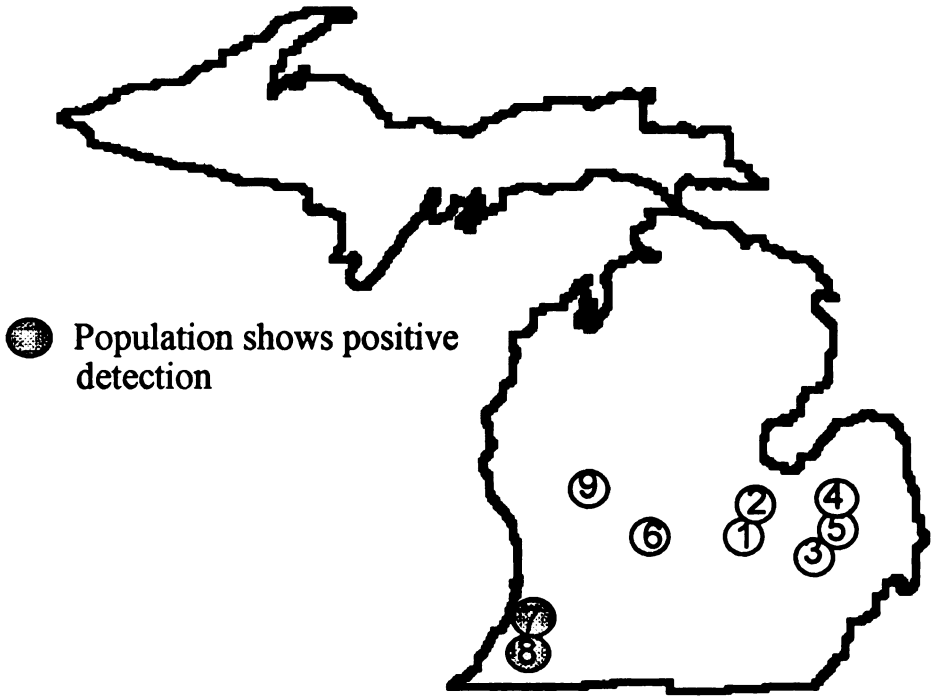


Figure III.5. Locations of nine populations of Colorado potato beetle tested with the RAPD primers for detecting *Bacillus thuringiensis* δ-endotoxin resistance. ①:Clio 1; ②:Clio 2; ③:Imlay1; ④:Imlay 2; ⑤: Imlay 3; ⑥:Lansing; ⑦:SWMREC; ⑧:BQ; ⑨:Lakeview

Mortality of 100% was observed in all nine locations. A total of 1000 field collected beetles were tested in pooled samples of fifty larva (20 pooled samples).

All of the twenty pooled samples tested carried the R-14 marker found in the laboratory colony resistance to *B. thuringiensis* CryIIIA δ-endotoxin. Assuming that each pooled samples carried at least one positive individual, therefore, at the minimum, 20 individuals of 1,000 are positive. This indicates that at least 0.02% the time the R-14 will give a positive detection.

Two of nine field sites sample tested showed the R-17 markers (Figure III.5). One site, SWMREC, was a new site for potato research and a Michigan State University,

Agricultural Experiment Station Research Farm. The potato has been planted for two seasons only. The other site, BQ, was a long-established commercial tomato production field. Further analysis of the pooled samples showed that 7 out of 250 and 2 out of 200 from SWMREC and BQ populations respectively, carried the markers. Overall, these positive samples represent 0.09% of the total populations tested.

DISCUSSION

The RAPD analysis carried out here was the first attempt to use this approach as a diagnostic tool for a resistant trait in field populations. Two primers (R-14 and R-17) tested have potential for CryIIIA δ-endotoxin resistance diagnostic probes. Primers R-17 co-segregate with the phenotype of *B. thuringiensis* CryIIIA δ-endotoxin resistance in our laboratory strain of Colorado potato beetles. The diagnostic fragment is linked to the resistance allele co-segregates with the dominant alleles and heterozygotes (R/S) can not be differentiate homozygous (R/R) genotype. This is similar to observations made by Landry et a. (1993). Therefore, without prior knowledge of the genetic characteristic of any individual, its genotype can not be determined solely based on the presence or absence of the R-17 RAPD-PCR diagnostic fragment.

The 650 basepairs fragment is amplified by R-14 and does not show significant linkage to the resistant allele. Using primer R-17, fragment 1,800 bp shows significant linkage to the resistant allele. Fragment 2,020 was amplified only in susceptible individuals. Figure III.3 show the diagram of the expected and observed phenotype in F_1 progenies from reciprocal cross. The R-17 primer may amplify linked fragments from different sites in respect to the tolerance trait in susceptible and resistant strains

The annealing site in the susceptible chromosome may be in a region of rapid change resulting from unequal crossing over or nucleotide slippage. This is known to occur commonly in the repetitive regions of other genomes. The annealing sites, which

are inverted repeats of the complementary sequence, occur frequently in the repetitive regions (Black et al. 1993, Levinson & Gutman 1987).

The disapearance of the susceptible marker in the F_1 progenies may be as a result of artifact of primer interference. Further test was done to determine the cause of this phenomenon. There was an interference in amplification of the 2020 bp fragment when both alleles present in heterozygote individuals. Modification of the RAPD buffer was done to avoid the interference.

Molecular markers are important tools for generating genetic linkage maps. The RAPD technique has proven to be the most sensitive and efficient method to generate, in a very short time, molecular markers for genetic mapping or fingerprinting (Mullis & Faloona 1987, Saiki et al. 1988, Reiter et al. 1992, Black et al 1993, Landry et al. 1993). No genetic linkage map of Colorado potato beetle has yet been constructed. The linkage map study reported herein is the first Colorado potato beetle map. The genetic map was constructed on the segregation of two marker types (RAPD and phenotype). A preliminary linkage analysis was done on the backcross progeny between F_1 heterozygotes and homozygous recessive parent. Assuming no sex limited crossing over and that the fragments are allelic markers, the progeny were scored as heterozygotes. If the amplified fragments were not linked, the observed segregation would in the F_1 backcross should fit a 1:1 Mendelion ratio. The R-17 primer tested here was not observed to be 1:1 thus showing linkage to the tolerance trait. These markers may amplify specific sites in respect to the gene for tolerance to *B. thuringiensis* CryIIIA δ-endotoxin.

Two RAPD-PCR diagnostic fragments for CryIIIA resistance were tested using resistant laboratory population, and were used to determine the frequency of the markers in limited field populations. R-14 primer can not useful in predicting the evolution of *B. thuringiensis* CryIIIA δ-endotoxin resistance because the marker is not linked to the resistance gene. The R-17 primer, however, shows promise for use in detection of resistance development in field populations. The significantly lower false detection

(0.09%) of the resistant allele in the field population, makes it possible to use the primer with reliability. More field populations and more individuals within population are needed to confirm the reliability of the diagnostic primer. The next logical step is to do a large scale field survey to determine the frequency of Colorado potato beetles carrying this marker or its allele in field populations in Michigan and perhaps nationwide. This survey will provide a base-line frequency from which to assess future changes that can be correlated with *B. thuringiensis* CryIIIA δ-endotoxin resistance development.

The individuals showing positive markers were collected from only two field populations: SWMREC and BQ. Both SWMREC and BQ sites were tomato fields for at least three years. In some parts of Northern America, Colorado potato beetle, is a serious insect pest of tomato (Latheef & Harcourt 1973, Schalk & Stoner 1979, Cantelo & Cantwell 1983). When the main host (potato) is exhausted, Colorado potato beetles will start emigrating to alternate host such as tomato (Latheef & Harcourt 1972, 1974). Studies by Latheef & Harcourt (1972) showed that tomato is a less suitable host for Colorado potato beetles. The presence of tomatine in most Solanaceae may deter the feeding activity of Colorado potato beetle.

Tomatine has been isolated from plants limited to the family of Solanaceae and, in particular, to the genera of *Solanum* and *Lycopersicon*. Tomato (*L. esculantum* Mill) and nightshade (*Solanum* spp.) are among *Lycopersicon* that produce tomatine. Potato, however, does not contain tomatine (Roddick 1974). Alkaloids of the potato plants (solanine) was reported to be less harmful to Colorado potato beetles than tomatine. Tomatine, a steroidal glycoalkaloid, is a potent Colorado potato beetle feeding deterrent (Roddick 1974, Sinden et al. 1978, Barbour & Kennedey 1991). Sturckow & Low (1961) demonstrated that Colorado potato beetle from wild populations were more tolerance to tomatine than the DDT-resistant beetles.

The toxicity of tomatine to a wide range of insects is well documented (Gallardo et al. 1990, Dimock et al. 1986, Chan & Tam 1985, Farrar & Kennedy 1990, Gallardo &

Boethel 1990), but the mechanism of toxicity is poorly understood. The mode of action of tomatine against Colorado potato beetle has been correlated with the ability of tomatine to alter the membrane integrity and cause lysis (Rodick & Drysdale 1984). In susceptible insects, cell lysis also resulted after the ingestion of *B. thrungiensis* δ-endotoxin. Therefore, Colorado potato beetles that have fed on tomato leaves and beetles fed on δ-endotoxin treated potato leaves might possibly exhibit similar adaptation. Both SWMREC and BQ populations possibly exhibit similar membrane mechanism to tolerate the high tomatine content and to detoxify the *B. thrungiensis* δ-endotoxin.

Colorado potato beetles from SWMREC and BQ were collected from the same county (Van Buren). Geographically, SWMREC is about 5 miles from the BQ population. Thus, they may be from the same evolutionary ancestor. The genetic variation based on the RAPD-PCR banding pattern is similar, and this also supports the genetic relatedness.

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APPENDIX III.1

Primers screened for developing DNA marker for gene/s responsible for resistance to *B. thuringiensis* δ-endotoxin in Colorado potato beetle

Primers screened for developing DNA marker for gene/s responsible for resistance to *B. thuringiensis* endotoxin in Colorado potato beetle

No.	Primer	Sequence 5' to 3'
1	OPB-01	GTTTCGCTCC
2	OPB-02	TGATCCCTGG
3	OPB-03	CATCCCCCTG
4	OPB-04	GGACTGGAGT
5	OPB-05	TGCGCCCTTC
6	OPB-06	TGCTCTGCCC
7	OPB-09	TGGGGGACTC
8	OPB-10	CTGCTGGGAC
9	OPB-12	CCTTGACGCA
10	OPB-13	TTCCCCCGCT
11	OPB-14	TCCGCTCTGG
12	OPB-16	TTTGCCCGGA
13	OPB-18	AGGGAACGCG
14	OPB-19	ACCCCCGAAG
15	OPB-20	GGACCCTTAC
16	OPC-01	TTCGAGCCAG
17	OPC-02	GTGAGGCGTC
18	OPC-03	GGGGGTCTTT
19	OPC-04	CCGCATCTAC
20	OPC-05	GATGACCGCC
21	OPC-06	GAACGGACTC
22	OPC-07	GTCCCGACGA
23	OPC-08	TGGACCGGTG
24	OPC-09	CTCACCGTCC
25	OPC-10	TGTCTGGGTG
26	OPC-11	AAAGCTGCGG
27	OPC-12	TGTCATCCCC
28	OPC-13	AAGCCTCGTC
29	OPC-14	TGCGTGCTTG
30	OPC-15	GACGGATCAG
31	OPC-16	CACACTCCAG
32	OPC-17	TTCCCCCCAG
33	OPC-18	TGAGTGGGTG
34	OPC-19	GTTGCCAGCC
35	OPC-20	ACTTCGCCAC
36	OPE-01	CCCAAGGTCC
37	OPE-02	GGTGCGGGAA

Continued

No.	Primer	Sequence 5' to 3'
38	OPE-03	CCAGATGCAC
39	OPE-04	GTGACATGCC
40	OPE-05	TCAGGGAGGT
41	OPE-06	AAGACCCCTC
42	OPE-07	AGATGCAGCC
43	OPE-08	TCACCACGGT
44	OPE-09	CTTCACCCGA
45	OPE-10	CACCAGGTGA
46	OPE-11	GAGTCTCAGG
47	OPE-12	TTATCGCCCC
48	OPE-13	CCCGATTCGG
49	OPE-14	TGCGGCTGAG
50	OPE-15	ACGCACAACC
51	OPE-16	GGTGACTGTG
52	OPE-17	CTACTGCCGT
53	OPE-18	GGACTGCAGA
54	OPE-19	ACGGCGTATG
55	OPE-20	AACGGTGACC
56	OPG-01	CTACGGAGGA
57	OPG-02	GTGAGGCGTC
58	OPG-03	GAGCCCTCCA
59	OPG-04	AGCGTGTCTG
60	OPG-05	CTGAGACGGA
61	OPG-06	GTGCCTAACC
62	OPG-07	GAACCTGCGG
63	OPG-08	TCACGTCCAC
64	OPG-09	CTGACCGTCC
65	OPG-10	AGGGCCGTCT
66	OPG-11	TGCCCGTCGT
67	OPG-12	CAGCTCACGA
68	OPG-13	CTCTCCGCCA
69	OPG-14	GGATGAGACC
70	OPG-15	ACTGGGACTC
71	OPG-16	AGCGTCCTCC
72	OPG-17	ACGACCGACA
73	OPG-18	GGCTCATGTG
74	OPG-19	GTCAGGGCAA
75	OPG-20	TCTCCCTCAG
76	OPI-01	ACCTGGACAC
77	OPI-02	GGAGGAGAGG

Continued

No.	Primer	Sequence 5' to 3'
78	OPI-03	CAGAAGCCCA
79	OPI-04	CCGCCTAGTC
80	OPI-05	TGTTCCACGG
81	OPI-06	AAGGCGGCAG
82	OPI-07	CAGCGACAAG
83	OPI-08	TTTGCCCGGT
84	OPI-09	TGGAGAGCAG
85	OPI-10	ACAACGCGAG
86	OPI-11	ACATGCCGTG
87	OPI-12	AGAGGGCACA
88	OPI-13	CTGGGGCTGA
89	OPI-14	TGACGGCGGT
90	OPI-15	TCATCCGAGG
91	OPI-16	TCTCCGCCCT
92	OPI-17	GGTGGTGATG
93	OPI-18	TGCCCAGCCT
94	OPI-19	AATGCGGGAG
95	OPI-20	AAAGTGCGGG
96	OPL-01	GGCATGACCT
97	OPL-02	TGGGCGTCAA
98	OPL-03	CCAGCAGCTT
99	OPL-04	GACTGCACAC
100	OPL-05	ACGCAGGCAC
101	OPL-06	GCGGGAAGAG
102	OPL-07	AGGCGGGAAC
103	OPL-08	AGCAGGTGGA
104	OPL-09	TGCGAGAGTC
105	OPL-10	TGGGAGATGG
106	OPL-11	ACGATGAGCC
107	OPL-12	GGGCGGTACT
108	OPL-13	ACCGCCTGCT
109	OPL-14	GTGACAGGCT
110	OPL-15	AAGAGAGGGG
111	OPL-16	AGGTTGCAGG
112	OPL-17	AGCCTGAGCC
113	OPL-18	ACCACCCACC
114	OPL-19	GAGTGGTGAC
115	OPL-20	TGGTGGACC
116	OPM-01	GTTGGTGGCT
117	OPM-02	ACCACGCCTC

Continued

No.	Primer	Sequence 5' to 3'
118	OPM-03	GGGGGATGAG
119	OPM-04	GGCGGTTGTC
120	OPM-06	CTGGGCAACT
121	OPM-07	CCGTGACTCA
122	OPM-08	TCTGTTCCCC
123	OPM-10	TCTGGCGCAC
124	OPM-11	GTCCACTGTG
125	OPM-14	AGGGTCGTTC
126	OPM-15	GACCTACCAC
127	OPM-16	GTAACCAGCC
128	OPM-17	TCAGTCCGGG
129	OPM-18	CACCATCCGT
130	OPM-20	AGGTCTTGGG
131	OPO-01	GGCATGACCT
132	OPO-02	TGGGCGTCAA
133	OPO-03	CCAGCAGCTT
134	OPO-04	GACTGCACAC
135	OPO-05	ACGCAGGCAC
136	OPO-06	GCGGGAAGAG
137	OPO-07	AGGCGGGAAC
138	OPO-08	AGCAGGTGGA
139	OPO-09	TGCGAGAGTC
140	OPO-10	TGGGAGATGG
141	OPO-11	ACGATGAGCC
142	OPO-12	GGGCGGTACT
143	OPO-13	ACCGCCTGCT
144	OPO-14	GTGACAGGCT
145	OPO-15	AAGAGAGGGG
146	OPO-16	AGGTTGCAGG
147	OPO-17	AGCCTGAGCC
148	OPO-18	ACCACCCACC
149	OPO-19	GAGTGGTGAC
150	OPO-20	TGGTGGACC
151	R-01	TGCCCCTCCT
152	R-02	CACAGCTGCC
153	R-03	ACACAGAGGG
154	R-04	CCCGTAGCAC
155	R-05	GACCTAGTGG
156	R-06	GTCTACGGCA
157	R-07	ACTGGCCTGA

Continued

No.	Primer	Sequence 5' to 3'
158	R-08	CCCGTTGCCT
159	R-09	TGAGCACGAG
160	R-10	CCATTCCCCA
161	R-11	GTAGCCGTCT
162	R-12	ACAGGTGCGT
163	R-13	GGACGACAAG
164	R-14	CAGGATTCCC
165	R-15	GGACAACGAG
166	R-16	CTCTGCGCGT
167	R-17	CCGTACGTAG
168	R-18	GGCTTTGCCA
169	R-19	CCTCCTCATC
170	R-20	ACGGCAAGGA
171	OPS-01	CTACTGCGCT
172	OPS-02	CCTCTGACTG
173	OPS-03	CAGAGGTCCC
174	OPS-04	CACCCCCTTG
175	OPS-05	TTTGGGGCCT
176	OPS-08	TTCAGGGTGG
177	OPS-09	TCCTGGTCCC
178	OPS-10	ACCGTTCCAG
179	OPS-11	AGTCGGGTGG
180	OPS-12	CTGGGTGAGT
181	OPS-13	GTCGTTCCTG
182	OPS-14	AAAGGGGTCC
183	OPS-15	CAGTTCACGG
184	OPS-17	TGGGGACCAC
185	OPS-18	CTGGCGAACT
186	OPS-19	GAGTCAGCAG
187	OPS-20	TCTGGACGGA
188	OPS-17	TGGGGACCAC
189	OPS-18	CTGGCGAACT
190	OPS-19	GAGTCAGCAG
191	OPS-20	TCTGGACGGA
192	OPT-01	GGGCCATCAT
193	OPT-02	GGAGAGACTC
194	OPT-03	TCCACTCCTG
195	OPT-04	CACAGAGGGA
196	OPT-05	GGGTTTGGCA
197	OPT-06	CAAGGGCAGA

Continued

No.	Primer	Sequence 5' to 3'
198	OPT-07	GGCAGGCTGT
199	OPT-08	AACGGCGACA
200	OPT-09	CACCCCTGAG
201	OPT-10	CCTTCGGAAG
202	OPT-11	TTCCCCGCGA
203	OPT-12	GGGTGTGTAG
204	OPT-13	AGGACTGCCA
205	OPT-14	AATGCCGCAG
206	OPT-15	GGATGCCACT
207	OPT-16	GGTGAACGCT
208	OPT-17	CCAACGTCGT
209	OPT-18	GATGCCAGAC
210	OPT-19	GTCCGTATGG
211	OPT-20	GACCAATGCC
212	OPU-01	ACGGACGTCA
213	OPU-02	CTGAGGTCTC
214	OPU-03	CTGAGGTCTC
215	OPU-04	ACCTTCGGAC
216	OPU-05	TTGGCGGCCT
217	OPU-07	CCTGCTCATC
218	OPU-08	GGCGAAGGTT
219	OPU-10	ACCTCGGCAC
220	OPU-11	AGACCCAGAG
221	OPU-12	TCACCAGCCA
222	OPU-13	GGCTGGTTCC
219	OPU-14	TGGGTCCCTC
220	OPU-15	ACGGGCCAGT
221	OPU-17	ACCTGGGGAG
222	OPU-18	GAGGTCCACA
223	OPU-19	GTCAGTGCGG
224	OPU-20	ACAGCCCCCA
225	OPV-01	TGACGCATGG
226	OPV-02	AGTCACTCCC
227	OPV-03	CTCCCTGCAA
228	OPV-04	CCCCTCACGA
229	OPV-05	TCCGAGAGGG
230	OPV-06	ACGCCCAGGT
231	OPV-07	GAAGCCAGCC
232	OPV-08	GGACGGCGTT

Continued

No.	Primer	Sequence 5' to 3'
233	OPV-09	TGTACCCGTC
234	OPV-10	GGACCTGCTG
235	OPV-11	CTCGACAGAG
236	OPV-12	ACCCCCCACT
237	OPV-13	ACCCCCTGAA
238	OPV-14	AGATCCCGCC
239	OPV-15	CAGTGCCGGT
240	OPV-16	ACACCCCACA
241	OPV-17	ACCGGCTTGT
242	OPV-18	TGGTGGCGTT
243	OPV-19	GGGTGTGCAG
244	OPV-20	CAGCATGGTC
245	OPW-01	CTCAGTGTCC
246	OPW-02	ACCCCGCCAA
247	OPW-03	GTCCGGAGTG
248	OPW-04	CAGAAGCGGA
249	OPW-05	GGCGGATAAG
250	OPW-06	AGGCCCGATG
251	OPW-07	CTGGACGTCA
252	OPW-08	GACTGCCTCT
253	OPW-09	GTGACCGAGT
254	OPW-10	TCGCATCCCT
255	OPW-11	CTGATGCGTG
256	OPW-12	TGGGCAGAAG
257	OPW-13	CACAGCGACA
258	OPW-14	CTGCTGAGCA
259	OPW-15	ACACCGGAAC
260	OPW-16	CAGCCTACCA
261	OPW-17	GTCCTGGGTT
262	OPW-18	TTCAGGGCAC
263	OPW-19	CAAAGCGCTC
264	OPW-20	TGTGGCAGCA
265	OPX-01	CTGGGCACGA
266	OPX-02	TTCCGCCACC
267	OPX-03	TGGCGCAGTG
268	OPX-04	CCGCTACCGA
269	OPX-05	CCTTTCCCTC
270	OPX-06	ACGCCAGAGG
271	OPX-07	GAGCGAGGCT
272	OPX-08	CAGGGGTGGA

Continued

No.	Primer	Sequence 5' to 3'
273	OPX-09	GGTCTGGTTG
274	OPX-10	CCCTAGACTG
275	OPX-11	GGAGCCTCAG
276	OPX-12	TCGCCAGCCA
277	OPX-13	ACGGGAGCCA
278	OPX-14	ACAGGTGCTG
279	OPX-15	CAGACAAGCC
280	OPX-16	CTCTGTTCGG
281	OPX-17	GACACGGACC
282	OPX-18	GACTAGGTGG
283	OPX-19	TGGCAAGGCA
284	OPX-15	CAGACAAGCC
285	OPX-16	CTCTGTTCGG
286	OPX-17	GACACGGACC
287	OPX-18	GACTAGGTGG
288	OPX-19	TGGCAAGGCA
289	OPX-20	CCCAGCTAGA
290	OPZ-01	TCTGTGCCAC
291	OPZ-02	CCTACGGGGA
292	OPZ-03	CAGCACCGCA
293	OPZ-04	AGGCTGTGCT
294	OPZ-05	TCCCATGCTG
295	OPZ-06	GTGCCGTTCA
296	OPZ-07	CCAGGAGGAC
297	OPZ-08	GGGTGGGTAA
298	OPZ-09	CACCCCAGTC
299	OPZ-10	CCGACAAACC
300	OPZ-11	CTCAGTCGCA
301	OPZ-12	TCAACGGGAC
302	OPZ-13	GACTAAGCCC
303	OPZ-14	TCGGAGGTTC
304	OPZ-15	CAGGGCTTTC
305	OPZ-16	TCCCCATCAC
306	OPZ-17	CCTTCCCACT
307	OPZ-18	AGGGTCTGTG
308	OPZ-19	GTGCGAGCAA
309	OPZ-20	ACTTTGGCGG

APPENDIX III.2

PCR-RAPD products of individual sample of Colorado potato beetle amplified by primer R-14

Sample	Band Number																	
No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1						+					+	+			+		+	
2						+												
3						+												
4	+					+			+		+							
5	+					+					+							
6															+			
7	+	+		+		+	+	+	+	+	+		+					
8						+		+	+	+	+		+		+			
9						+				+	+				+			
10					+	+				+	+				+			
11						+		+			+				+			
12						+		+			+				+			
13						+		+		+	+				+			
14	+					+					+							
15	+					+					+	+						
16						+									+			
17												+			+			
18						+					+				+			
19						+		+			+	+			+			
20						+					+							
21						+		+			+	+			+			
22						+			+	+	+				+			
23	+				+	+					+				+			
24						+			+	+	+				+			
25					+	+	+	+	+	+								
26					+	+	+	+		+	+	+			+			
27	+	+	+			+	+	+	+	+	+	+						
28					+	+		+	+	+	+	+			+			
29					+	+			+	+	+							
30	+	+					+	+	+	+	+	+			+			
31	+					+				+	+				+			
32	+					+			+	+	+				+			
33						+					+	+			+			
34					+	+					+	+			+			
35					+	+												
36	+					+					+							
37	+					+									+			
38	+														+			
39	+					+									+			
40	+					+								+	+			

Sample	Band Number																	
No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
41	+					+	+								+			
42						+												
43						+									+	+		
44						+												
45						+									+			
46						+									+	+		
47						+									+			
48						+										+		
49				+		+		+						+	+			
50	+					+						+			+			
51				+		+				+	+	+			+			
52											+							
53	+					+			+	+	+	+						
54	+					+					+							
55						+												
56						+					+							
57						+					+							
58					+	+												
59						+												
60						+												
61						+												
62					+	+												
63						+					+				+			
64		+			+						+				+			
65	+				+						+	+			+			
66	+				+													
67						+												
68						+				+	+			+				
69						+				+	+	+						
70						+				+	+	+			+			
71						+				+	+	+			+			
72											+	+		+				
73						+					+	+			+		+	
74						+			+		+		+		+		+	
75						+			+					+				
76						+		+			+	+			+		+	
77				+	+	+		+			+	+			+		+	
78								+			+	+					+	
79						+		+			+	+	+	+	+		+	
80						+		+			+		+		+		+	

Sample	Band Number																	
No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
81	+		+	+	+	+		+			+	+			+		+	
82	+					+									+			
83	+				+	+		+							+			
84	+		+												+			
85	+		+			+									+			
86			+			+									+			
87	+		+	+											+	+		
88						+		+			+	+						
89	+					+					+							
90						+									+			
91											+				+			
92						+												
93	+		+	+		+					+							
94						+												
95						+												
96	+					+					+							
97					+	+	+				+							
98	+					+					+							
99	+					+												
100						+				+								
101						+					+				+			
102											+				+	+		
103						+	+		+		+							
104											+			+				
105	+					+				+								
106						+								+	+			
107						+				+	+				+			
108						+					+				+			
109						+					+				+			
110						+					+	+			+			
111										+	+							
112						+				+								
113						+				+	+				+			
114						+				+	+				+			
115						+					+				+			
116						+				+	+							
117						+						+						
118	+	+	+	+	+	+		+		+	+	+			+		+	
119										+	+		+					
120					+	+					+	+		+	+		+	

121									+	+			+			+		
122									+	+			+			+		
Total +	33	5	8	8	19	93	8	22	21	33	74	30	8	9	68	7	11	0
Total -	89	17	14	114	103	29	114	00	101	89	48	92	114	113	74	115	111	122

[a] The number of individuals used for PCR-RAPD analysis
[b] Band number 1 to 18 indicate the bands from larger to smaller amplified by primer

APPENDIX III.3

PCR-RAPD products of individual sample of Colorado potato beetle amplified by primer R-17

Sample	Locus Number:										
No.	1	2	3	4	5	6	7	8	9	10	11
1		+			+	+	+				
2	+	+			+	+					
3		+	+	+							
4		+		+	+						
5	+	+			+						
6							+	+			
7					+		+	+			
8			+								
9					+				+		
10					+	+	+				
11	+				+					+	
12			+		+						
13	+				+				+		+
14		+			+						
15	+	+			+				+		
16						+	+	+	+		
17	+	+		+							
18					+					+	
19	+	+	+	+	+					+	
20		+	+		+						
21	+	+			+				+		
22			+	+							
23				+	+	+			+		
24		+	+								
25					+						
26					+						
27					+			+			
28			+								
29			+			+	+	+	+		
30					+	+			+		
31								+			
32			+				+	+			+
33					+	+		+			
34	+		+					+			
35		+	+	+	+		+				
36			+	+	+	+	+	+		+	
37			+	+	+						
38						+	+	+			
39		+	+			+	+	+			
40	+		+			+		+			

Sample	Locus Number:										
No.	1	2	3	4	5	6	7	8	9	10	11
41					+						
42					+						
43					+						
44					+						
45					+						
46					+						
47					+						
48					+						
49					+						
50	+				+	+					
51			+			+		+	+		
52					+						
53	+				+						
54			+			+		+	+		
55					+						
56	+				+						
57	+		+		+		+	+		+	
58	+				+		+	+		+	
59	+				+		+	+			
60	+	+	+								
61	+	+			+						
62					+						
63		+					+				
64	+				+	+					
65					+				+		
66					+						
67					+		+		+		
68				+	+	+			+		
69	+	+			+						
70	+				+						
71		+				+			+		
72	+				+						
73	+				+						
74					+	+					
75						+		+			
76	+		+				+				
77					+			+			
78	+	+								+	
79	+		+							+	
80					+			+			

Sample	Locus Number:										
No.	1	2	3	4	5	6	7	8	9	10	11
81	+		+					+			
82	+	+			+			+			
83		+		+	+	+		+			
84					+			+			
85	+	+			+			+			
86	+				+			+			
87	+	+	+						+		
88					+			+			
89	+				+			+			
90	+				+				+		
91	+				+		+	+			
92				+	+	+		+	+		
93	+		+		+	+	+	+	+		
94	+		+		+	+	+				
95	+				+	+					
96	+				+			+			
97	+				+			+			
98	+				+		+		+		
99	+		+	+							
100	+				+			+			
101				+	+	+			+		
102				+	+			+			
103				+	+			+	+	+	
104				+	+	+			+		
105	+				+			+			
106			+		+			+			
107	+				+			+			
108	+				+			+			
109	+				+			+			
110	+				+			+			
111	+			+	+						
112	+				+	+		+			
113	+			+	+		+	+			
114	+			+	+			+			
115	+				+			+			
116	+				+						
117	+			+	+	+	+				
118	+				+	+			+	+	
119				+	+	+					
120	+			+	+	+	+				

121				+	+	+		+			
122				+	+	+		+			
Total +	56	24	28	25	95	35	24	49	23	10	2
Total -	66	98	94	97	27	87	98	73	99	112	10

[a] The number of individuals used for PCR-RAPD analysis
[b] Band number 1 to 11 indicate the bands from larger to smaller amplified by primer

CHAPTER IV

Isolation And Identification Of mRNA Conferring Resistance To *Bacillus thuringiensis* CryIIIA δ-endotoxin In Colorado Potato Beetle (Coleoptera: Chrysomelidae)

INTRODUCTION

Insecticide resistance is one of the most important problems facing modern plant and health protection. The number of resistant insect and mite species continues to grow annually, and already more than 500 species have acquired resistance (Georghiou 1990). The recent discovery that several important species of pest insects have the capacity to evolve resistance to *Bacillus thuringiensis* δ-endotoxins now raises questions regarding the long-term durability of this biological insecticide in pest control. These questions of durability are especially critical in the rapidly advancing area of plant genetic transformation, which currently is focusing primarily on the use of *B. thuringiensis* δ-endotoxins genes to impart pest resistance in several major crop species (Gasser & Fraley 1989, Boulter *et al.* 1990, Brunke & Meeusen 1991). The widespread development of pest resistance could seriously diminish the economic value of this technological development and force continued reliance on chemical insecticides (Gould 1988a, 1988b). Within the last few years, 8 species have been selected for resistance to *B. thuringiensis* δ-endotoxins (McGaughey & Whalon 1992).

Resistance in the Colorado potato beetle, *Leptinotarsa decemlineata* Say, has been reported only from laboratory selection experiments, but the capacity for resistance in this species is of concern because of the great economic significance of the pest. The mechanism of resistance in *L. decemlineata*, however, is unknown at this time. The emerging understanding *B. thuringiensis* δ-endotoxin's possible mode of action has guided scientists to develop several hypotheses for the mechanism of resistance to this δ-endotoxin in insect pests. However, most of these studies have been done on Lepidoptera

(Endo & Nishiitsutsuji-Uwo 1980, Sacchi et al. 1986, Knowles & Ellar 1987, Hofte & Whiteley 1989).

Three steps are thought to be involved in the mechanism of action of the crystal proteins of *B. thuringiensis* (Lilley et al. 1980, English & Slatin 1992, Knowels & Dow 1993). The protein is dissolved in the gut and then activited by means of gut proteases of the susceptible insect. The activated protein appears to have specific interaction with the brush border membrane (Slatin et al. 1990). In a study by Bravo et al. (1992) Colorado potato beetles exposed to δ-endotoxin were found to accumulate the CryIIIA δ-endotoxin in the microvilli of the epithalial cells at the posterior part of the midgut. The involvement of receptor proteins in this interaction has been demonstrated in Lepidoptera by several researches (Hofmann et al. 1988, Van Rie et al 1990, Ferre et al. 1991). Altered binding affinity of receptor proteins is one of the most widely hypothesized mechanism of insect resistance to *B. thuringiensis* δ-endotoxin (Van Rie et al. 1990).

The final step in intoxication occurs with the formation of pores in the plasma membrane. After the δ-endotoxin binds to receptors, the membrane internalize the hydrophobic surfaces of the crystal. The internalization process leads the of CryIIIA δ-endotoxin in to close contact with the membrane resulting in conformation changes (Li et al. 1991). The resulting conformation changes might be responsible for the forming of pores or channels in a plannar lipid bilayer or membrane. This leads to disruption of the permeability barrier of the membrane, leakage of K^+ and H_2O, cell lysis and breakdown of the gut integrity.

The complex mode of action of the δ-endotoxin leads one to speculate on several possible mechanisms of resistance in Colorado potato beetle including decrease in δ-endotoxin activity (assuming that protease is critical for CryIII δ-endotoxin activation), altered receptors, corrupted conformation of crystal, and/or recovery.

Any or a combination of these hypothesized resistance mechanisms are possible in Colorado potato beetle. In addition, these mechanisms are very likely involved with

specific proteins that are differentially expressed in the susceptible and resistant strains. Comparison of protein population resulting from variant gene expression in the different colonies of Colorado potato beetle could provide a critical information of the proteins that are involved in the resistance mechanism.

An effective method to screen different protein is called mRNA Differential Display System, and it has been developed to identify and isolate those genes that are differentially expressed in various cells or the same cells under different conditions (Liang & Pardee 1992). This technique involves the reverse transcriptation of the mRNA followed by Polymerase Chain Reaction (PCR) in the presence of a 10-mers primer. I have done several experiments using the above method to determine whether there is difference in RNApopulations between the susceptible and resistant Colorado potato beetle strains. The goal of this study is to identify and isolate the gene/s conferring resistance to *B. thuringiensis* δ-endotoxin in *L. decemlineata* by means of midgut mRNAs comparative analysis. Once the gene/s conferring resistance to *B. thuringiensis* δ-endotoxin is identified, beside enabling us to identify the mechanism of resistance, we can also develop detection system for resistance development in field populations by means of DNA marker.

OBJECTIVES

1. To determine the different RNA populations between susceptible and resistant colonies of Colorado potato beetle
2. To isolate cDNA encoding protein that is unique for either susceptible or resistance colony.
3. To determine the sequence of the specific cDNAs involved.

MATERIALS AND METHODS

Insect Rearing and Selection

Colorado potato beetle, *Leptinotarsa decemlineata* (Say), from 7 different field populations was initially selected and the survivors were brought to the laboratory and subsequently selected in every generation (Whalon et al. 1993). Second instars were selected with CryIII δ-endotoxin for over 29 generation resulting in over 200 fold resistance (Rahardja & Whalon 1994). Analysis of probit mortality lines from the F_1 reciprocal crosses indicated that *B. thuringiensis* δ-endotoxin resistance was inherited autosomaly and there were no maternal effects. The degree of dominance (D) was estimated to be 0.76 and 0.77 for the (Resistant X Susceptible) and (Susceptible X Resistant) F_1 generations respectively, indicating that *B. thuringiensis* CryIIIA δ-endotoxin resistance is conferred by partialy dominant gene(s) with additional influence of minor genes.

RNA Extraction

The isolation of total RNA was performed following the procedure provided by the manufacture (Promega). Midguts from 200-300 larvae (≈ 1 gram) from pooled susceptible and pooled resistant colonies were used for RNA extraction. The larvae were exposed to *B. thuringiensis* CryIIIA δ-endotoxin (LC_{50} for susceptible colony) prior to extraction. The tissue was denatured then disrupted with a high speed homogenizer for 15-30 sec. The RNA was separated from protein and other macromolecules by phenol extraction and precipitated incubating the RNA in equal volume of isoproponal overnight. Precipitated RNA was collected by centrifugation and the pellet was washed with ice-cold 75% ethanol. The RNA pellet was dried in a vacuum desiccator and resuspended in RNAse free water at -20 °C (no longer than 3 weeks) until use. All the RNA extraction procedures were carried out under RNAse free conditions.

mRNA Differential Display System

The mRNA Differential Display method was dcarried out using RNAmapTM kit (Gene Hunter Corporation). Reverse transcription reactions for RNA samples were performed in 20 µl final volume containing 5'-primer and the primer for the poly(A) tail (Tabel 1). The mixture then was incubated at 65 °C for 5 min, 37 °C for 60 min and 95 °C for 5 min. Reverse transcriptase was added to the mixture 10 min after incubation at 37 °C. The resulting complementary DNA (cDNA) was amplified using a second set of primers (AP-primers and 5'-primers) in a final volume of 20 µl containing the appropriate buffer, nucleotides, ^{35}S-dATP and Taq polymerase.

Amplification was run under the following condition: 94 °C for 30 second, 40 °C for 2 minutes, 72 °C for 30 second, for 40 cycles and then 72 °C for 5 minutes. The resulting amplification products were separated by electrophoresis on a 6% polyacrylimide gel. The gel was lifted from the gel apparatus with filter paper, dried, and used to expose film for 12-48 hours.

RESULTS

Twenty five different combinations of primers (Table 1) were examined in this study. RNA from 1 g of larvae of pooled susceptible and pooled resistant colonies was separately isolated. The pooled total RNA from the beetles were used to screened all the twenty five set of primers. All 25 sets of primers exhibited polymorphism in the sizes of the amplified cDNA fragments. However, most of the fragments are not uniquely expressed in either susceptible nor resistant strain of Colorado potato beetle.

Two sets of the primers exhibited polymorphims that related to the resistance to *B. thuringiensis* CryIIIA δ-endotoxin in Colorado potato beetle. AP-11 and T_{12}MG primers produced two unique fragments in the resistant colony. The second primers, AP-12 and T_{12}MA also yielded two specific fragments two the susceptible colony (Figure IV.1).

These unique fragments were further isolated and re-amplified twice with the same set of primers used during the first amplification. The fragment size ranged from 200 to 500 bases.

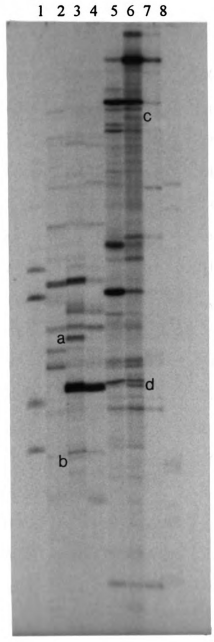

Figure IV. 1. Differential display of total RNA isolated from *Bacillus thuringiensis* δ-endotoxin susceptible and resistant Colorado potato beetles. 3 and 5 : resistant samples, 4 and 6: susceptible samples. a, b, c and d are unique fragments.

DISCUSSION

Reverse Transcript Polymerase Chain Reaction (RT-PCR) is a method to identify differentially expressed genes among thousands of individual mRNAs. In this study, the general strategy was to amplify partial cDNA sequences from subsets of mRNAs from both *Bacillus thuringiensis* δ-endotoxin susceptible and resistant Colorado potato beetles. The different sizes of amplified products resulted from the annealing positions of 5' primer to cDNA. These annealing positions were randomly distributed in distance from poly(A) tail. The present-absent display of cDNA from both susceptible and resistant colonies demonstrated in this study was an important preliminary finding. It may be a result of nucleiotide alterations, deletions or additions in the genome. Difference in transcriptional control may also be responsible for these specific patterns of mRNA observed.

Further studies are needed to reveal the nucleotide sequence of the putitive of diagnostic fragments yielded by primers AP-11 - $T_{12}MG$ and AP-12 - $T_{12}MA$. Once the nucleotide sequence is known, the sequence of a partial protein involved in the resistance mechanism in Colorado potato beetle may be resolved.

mRNA differential display has several technical advantages as compared to the RAPD technique. It is less speculative and it compares gene expression in the same cells of different strains. It is also much quicker, chromosome walking is not necessary to isolate the genes of interest.

Tabel IV.1. 5'-primers and 3'-primers used for identification and isolation of mRNA conferring resistance to *Bacillus thuringiensis* δ-endotoxin in Colorado potato beetle

NO.	5'-PRIMER	3'-PRIMER[a]
1	AP-11: 5'-CAGACCGTTC-3'	$T_{12}MA$
2	AP-11: 5'-CAGACCGTTC-3'	$T_{12}MC$
3	AP-11: 5'-CAGACCGTTC-3'	$T_{12}MT$
4	AP-11: 5'-CAGACCGTTC-3'	$T_{12}MG$
5	AP-12: 5'-TGCTGACCTG-3'	$T_{12}MA$
6	AP-12: 5'-TGCTGACCTG-3'	$T_{12}MC$
7	AP-12: 5'-TGCTGACCTG-3'	$T_{12}MT$
8	AP-12: 5'-TGCTGACCTG-3'	$T_{12}MG$
9	AP-15: 5'-AGGGCCTGTT-3'	$T_{12}MA$
10	AP-15: 5'-AGGGCCTGTT-3'	$T_{12}MC$
11	AP-15: 5'-AGGGCCTGTT-3'	$T_{12}MT$
12	AP-15: 5'-AGGGCCTGTT-3'	$T_{12}MG$
13	AP-13: 5'-AGTTAGGCAC-3'	$T_{12}MA$
14	AP-13: 5'-AGTTAGGCAC-3'	$T_{12}MC$
15	AP-13: 5'-AGTTAGGCAC-3'	$T_{12}MT$
16	AP-13: 5'-AGTTAGGCAC-3'	$T_{12}MG$
17	AP-14: 5'-AATGGGCTGA-3'	$T_{12}MA$
18	AP-14: 5'-AATGGGCTGA-3'	$T_{12}MC$
19	AP-14: 5'-AATGGGCTGA-3'	$T_{12}MT$
20	AP-14: 5'-AATGGGCTGA-3'	$T_{12}MG$

[a] M= A or C or G

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SUMMARY AND GENERAL CONCLUSION

The ability of Colorado potato beetle *Leptinotarsa decemlineata* (Say) to tolerate *Bacillus thuringiensis* CryIIIA δ-endotoxin has been demonstrated. This development raises questions regarding the long-term durability of this biological insecticide in potato pest control. Although the genetic basis of laboratory-selected resistance to *B. thuringiensis* CryIIIA δ-endotoxin may differ from resistance selected in the field, the laboratory data may be useful in devising strategies to hinder resistance evolution. The objective of this study was to examine inheritance and realized heritability of *B. thuringiensis* CryIIIA δ-endotoxin in Colorado potato beetle and to develop DNA markers for detection of genes conferring the resistance in field populations.

The resistance to *B. thuringiensis* CryIIIA δ-endotoxin in Colorado potato beetle was inherited autosomaly without maternal effects. The estimated degree of dominance (D) were 0.77 and 0.76 for the (R X S) and (S X R) F_1 generations, respectively, indicating that *B. thuringiensis* CryIIIA δ-endotoxin resistance is conferred by partially dominant genes. χ^2 analysis of mortality responses of backcrossed offspring suggested that resistance might be caused by more than one genes. When the selection pressure was removed, the resistance level of the selected colony decreased after five generations. However, the resistance level did not decrease further when the selection was removed for over 12 generations, but was still significantly higher (40-100 fold resistance) than the unselected colony. The discriminating dose (≈60 mg CryIIIA δ-endotoxin per liter of water, which kills 100% of the susceptible larvae) failed to eliminate this colony.

The realized heritability of Colorado potato beetle estimated for over 29 generations reached the highest value at generation four, and then decreased and reached values with very little fluctuation after the tenth generation. At generation 29, the

heritability estimates increased as did the percentage of adult emergence. The heritability estimates in the first 12 generations showed significant correlation with the increment of the resistance ratio. However, subsequent decrease of heritability estimates was not followed by a corresponding decline in resistance ratio nor did they correspond with a major shift in resistance gain occurred between the G_3-G_4, G_7-G_8, G_{11}-G_{12} and G_{16}-G_{17}. The mean estimated heritability value after 29 generations was relatively low (h^2 = 0.10).

There was no significant correlation between standard deviation of LC_{50} and the generation number over 29 generations in either the selected and unselected strains. However, the variance did appear to decrease slowly throughout the experiment in both strains. In general, genetic variance decreases as a result of directional selection, yet no significant elimination of genetic variance has occured under 34 generations of selection. The slope of the regression line between responses to selection and cumulative selection differential was relatively small indicating that the rate of the selection gain was slow and that the selection limits have not been reached after 29 generations of selection.

The projection of selection gains of 10 fold in LC_{50} predicted based on h^2 of the fourth generation (= 0.21) and selection pressure of 95% was in agreement with the observation indicating that selection gains can be predicted fairly well for several initial selections if the heritability of the trait is known.

Diagnostic fragments for gene(s) conferring *B. thuringiensis* CryIIIA δ-endotoxin resistance in laboratory reared Colorado potato beetles were identified by Polymerase Chain Reaction (PCR) analysis. Primers R-14 (5'-ACAGGTGCTG-3') and R-17 (5'-CCGTACGTAG-3') yielded 650 and 1800 base pairs genetic markers, respectively, for the resistant laboratory colony. These markers were linked to the gene(s) conferring resistance to the CryIIIA δ-endotoxin. The recombination fractions of 650 and 1800 base pairs genetic markers were 0.46 and 0.20, respectively. Although the markers are linked,

the distance in terms of DNA base pairs may be substantially far apart. The χ^2 for marker 650 bp indicates that the marker is randomly associated with the resistance gene or genes. The 1,800 bp, however, non randomly associates with the resistance phenotype. Because of the moderate linkage between resistance on marker R-17, the PCR fragment can be used as a starting point to isolate the gene for resistance itself.

Populations of Colorado potato beetle from nine different locations in Michigan were used to test the usefulness of the primers (R-14 and R-17) for field detection. Mortality of 100% was observed in all nine locations when 30 - 90 second instars were exposed to 50% of the discriminating dose. Total of 1000 field sampled beetles were tested in pooled samples of fifty larva (20 pooled samples).

All of the twenty pooled samples tested with R-14 carried the diagnostic marker fragment for resistance to *B. thuringiensis* CryIIIA δ-endotoxin. This indicates that at least 0.02% of the time the R-14 fragment will give a positive detection.

Two of nine field sites sample tested with R-17 showed the diagnostic markers. Further analysis of the pooled samples showed that 7 out of 250 and 2 out of 200 from SWMREC and BQ populations respectively, carried the markers. Overall, these positive samples represent 0.09% of the total populations tested.

Differentially expressed mRNA from resistant and susceptible Colorado potato beetle identified Reverse-Transcript-Polymerase Chain Reaction (RT-PCR) mRNAs. Ten different 10 to 15 oligomer primers used in twenty five different combinations of primers revealed polymorphism in the sizes of the amplified cDNA fragments. However, most of the fragments are not diagnostic for the resistance to *B. thuringiensis* CryIIIA δ-endotoxin in laboratory selected Colorado potato beetle.

Polymorphisms exhibited by two sets of primers were related to the resistance phenotype in Colorado potato beetle. AP-11 (5'-CAGACCGTTC-3') and T_{12}MG (a mixture of 3'-T_{12}AG-5', 3'-T_{12}CG-5', and 3'-T_{12}GG-5') primers produced two unique

fragments from RNA extracted from resistant larvae. The second set of primers, AP-12 (5'-TGCTGACCTG-3') and T_{12}MA (a mixture of 3'-T_{12}AA-5', 3'-T_{12}CA-5', and 3'-T_{12}GA-5'), also yielded two specific fragments using RNA from susceptible larvae. Further experiments are needed to determine the amino acid sequence of the unique fragments.

The study of the laboratory development of *B. thuringiensis* CryIIIA δ-endotoxin resistance in Colorado potato beetle provides an opportunity to develop control strategies before the resistance can occurs in a field population. If a similar resistance was to develop in field populations of Colorado potato beetle, reduced selection pressure would likely be a fruitful strategy to slow the evaluation of resistance because the population reverts back toward susceptibility. However, the presence of a stable, autosomaly inherited, dominant *B. thuringiensis* δ-endotoxin gene conferring 40 to 100 fold resistance could be very difficult to manage where this level of resistance could impart field survival without the presence of minor genes.

The mean realized heritability value of resistance to *B. thuringiensis* δ-endotoxin was relatively low. Selection gains can be predicted fairly well for several initial selected generations if the heritability of the trait is known. The projected response to selection increases as heritability value and selection pressure or intensity increased.

Assuming that similar resistance to *B. thuringiensis* CryIIIA δ-endotoxin develops in the field, the information of the inheritance and the estimates of h^2 of resistance to *B. thuringiensis* CryIIIA δ-endotoxin in laboratory Colorado potato beetle reported here should provide guidance to assess the risk of resistance development. The discriminating dose would give an early indication of whether the genes for resistance have reached the detectable frequency. The frequency of the genes could be more accurately detected with the more reliable but expensive DNA marker.

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