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# POTATO TUBERWORM (LEPIDOPTERA: GELICHIIDAE) RESISTANCE IN POTATO LINES WITH THE *Bacillus thuringiensis-cry1ac* GENE AND NATURAL RESISTANCE FACTORS

presented by

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has been accepted towards fulfillment
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Major Professor's Signature

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Date

# POTATO TUBERWORM (LEPIDOPTERA: GELICHIIDAE) RESISTANCE IN POTATO LINES WITH THE *Bacillus thuringiensis-cry1ac* GENE AND NATURAL RESISTANCE FACTORS

**By**

**Maria Allasas Estrada**

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## ABSTRACT

## POTATO TUBERWORM (LEPIDOPTERA: GELICHIIDAE) RESISTANCE IN POTATO LINES WITH THE *Bacillus thuringiensis-cry1ac* GENE AND NATURAL RESISTANCE FACTORS

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The potato tuberworm (*Phthorimaea operculella* (Zeller)) is one of the most common and destructive insect pests to potato (*Solanum tuberosum* L.). Previous host plant resistance work has not produced potato material that has appreciable levels of resistance to potato tuberworm. A potential solution to improve cultivated potato resistance to potato tuberworm is through combining resistance mechanisms of glycoalkaloids and glandular trichomes with *cry* genes. The objectives of this study were: 1) transform three potato lines which differ in natural host plant resistance with a codon-modified *Bt-cry1ac* gene; 2) verify insertion of *Bt-cry 1ac*, quantify the protein expression, and determine the number of inserted copies of the gene; and 3) conduct laboratory bioassays to test the potato tuberworm response on *Bt- cry Iac* transgenic lines. Putative transgenic lines of Spunta, ND5873-15 & NY123, were developed using vector construct (pSPUD 15) with the codon-modified *Bt-cry1ac* gene. Integration of *Bt-cry1ac* gene in Spunta and ND5873-15 transgenic lines was confirmed by the PCR and Southern blot analysis. Protein expression in the transgenic lines (0-0.58 ng/mg) was confirmed by ELISA. Detached leaf bioassays with neonate larvae of potato tuberworm showed that *cry1ac* gene was effective in controlling potato tuberworm 1$^{st}$ instar larvae (up to 97 % mortality). Combining natural host plant resistance and engineered resistance with the *Bacillus thuringiensis-cry1ac* gene in potato plant was effective in conferring increased resistance to potato tuberworm.

***I would like to dedicate this work to my loving husband Dexter, my little angel John Benedict, to my family in the Philippines Tita Belle, Tito Boyet, Tatay JR & Ate Imee, and in memory of my mother Edna.***

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# INTRODUCTION

## POTATO

Potato (*Solanum tuberosum* L.) is one of the most important crops for human nutrition worldwide and is a healthy source of carbohydrates, high quality protein, essential vitamins, minerals, and trace elements (Flanders et al. 1999). It is the highest ranking vegetable crop in production in the USA (Douches et al. 1996). Estimated production is ca. 300 million tons per year (Gebhardt et al. 2001). The three main targets of potato breeding are for fresh food market, processing industry, and for non-food industrial uses. At present, potato breeders are giving more emphasis in meeting both the quality requirements of processors and supermarkets, and the yield potential to ensure the commercial success of the variety they are releasing. In addition, they are also focusing their breeding efforts toward developing varieties that are resistant to insect pests and diseases. Potato is vegetatively or clonally propagated crop and is therefore vulnerable to many diseases and insect pests that affect both the foliage and the tuber (Gebhardt et al. 2001). Furthermore, potato has numerous pest and pathogens that can reduce yields and overall plant vigor (Coombs et al. 2001).

The present trend in potato breeding is combining quality with resistance to insect pests and diseases. Priorities are now being given toward resistance breeding to potato cyst nematode, late blight, blackleg and powdery scab, blemish diseases, storage diseases, viruses, and some of the most common destructive and most important insect pests: Colorado potato beetle, potato tuberworm and European corn borer (Naimov et al. 2003).

## POTATO TUBERWORM

One of the most common and destructive insect pests to potato worldwide, both in tropical and subtropical areas, is the potato tuberworm (*Phthorimaea operculella* (Zeller)) (Flanders et al. 1999). Potato tuberworm causes damage in both field and storage (Westedt et al. 1998), reduces the quality of produce, and increases the risk of pathogen infection. Furthermore, damage caused by potato tuberworm can tremendously reduce the potato yield because it attacks both the foliage and the tuber (Capinera 2001). In warmer climates, the quantity and quality losses in storage can be as high as 100% (Lagnaoui et al. 2000). Potato tuberworm life cycle, feeding habit and its ability to develop resistance to chemical insecticides is an increasing agricultural problem in the tropical and subtropical areas in the world (Naimov et al. 2003). Potato tuberworm spend their larval stages either in the foliage or in the tuber. The larvae mine both the foliage and the petiole creating transparent leaf blisters. Damage in the foliage caused by potato tuberworm may be sufficient to cause severe damage or death to the plant. The eggs that were oviposited on the exposed potato tubers will hatch and will then enter through the "eyes". They will make slender tunnels throughout the tuber and introduce bacterial rots. Mounds of frass at the tunnel entrances can give indication of an infested tuber.

## HOST PLANT RESISTANCE

Varying levels of resistance to insects occur naturally in crop plants and closely related species (Stoner 1996). Insect resistance appears to be a primitive trait in wild potatoes (Flanders et al. 1999).

*Solanum spp.* exhibit a wide variety of defense mechanisms against insect pests. The two general types of host plant resistance that can be found in *Solanum* are high

levels of glycoalkaloids and glandular trichomes. Potatoes contain glycoalkaloids that have long been known to possess antimicrobial and insecticidal properties (Tingey 1984). The glycoalkaloids present in the leaves can also act as insecticidal compounds. The two most common glycoalkaloids found in potatoes are α-chaconine and α-solanine, together they comprise as much as 95% of the total glycoalkaloid present in the potato (Lachman et al. 2001). Chemically, α-chaconine and α-solanine, are classified as steroidal glycoalkaloids. *Solanum* steroidal glycoalkaloids are large biologically-active secondary metabolites that have been isolated from more than 350 plant species (Lawson et al. 1993, Roddick 1986, Ripperger & Schreiber 1981). These are toxins that occur naturally in many edible and non-edible members of the *Solanaceae*, such as eggplant (*Solanum melongena*) and potato (*Solanum tuberosum*). Presence of steroidal glycoalkaloids in potato could both be a benefit and a concern (Lawson et al. 1993). Consumption of tubers with high steroidal glycoalkaloids concentration can be detrimental to the health of human and animals. The *Solanum* glycoalkaloids are cholinesterase inhibitors which result in neural function impairment (Hopkins 1995, Lachman et al. 2001). The fatal oral dose for an adult would be 420 mg (Lachman et al. 2001). Glycoalkaloids concentration in potato plants can be highly affected by the temperature. This explains why these protective glycoalkaloids are present at higher concentration in the aerial ( leaves, stems and sprouts) part of the potato plant and are normally present in lower concentration in the tubers (Lachman et al. 2001). Similarly, Lafta and Lorenzen (2000) observed a significant increase in foliar glycoalkaloids when plants were grown at higher temperature (32/27 °C).

Potato possesses another type of defense mechanism classified as glandular trichomes. Wild species of potato have glandular trichomes that confer resistance to

different kinds of insect pests. The most common types of glandular trichomes that can be found on the potato foliage are the type A and type B. A recently discovered wild potato species, *S. neocardenasii* Hawkes & Hjerting (series Tuberosa), from central Bolivia were observed to has glandular hairs in the foliage (Lapointe et al. 1986, Hawkes et al. 1983). This Bolivian wild potato is resistant to numerous insect pests like the potato tuberworm complex. Type A and type B glandular trichomes are associated with insect resistance in *S. berthaultii* (Yencho et al. 1994, Tingey 1991). Resistance of *S. berthaultii* to insects is associated with chemical factors localized in the glandular trichomes, in addition, a feeding deterrent is present in the trichomes (Tingey et al. 1994). Previous studies of droplets on the B trichomes showed that the inheritance of this character is inherited as a single dominant gene (Kalazich 1991, Gibson 1979).

Although, potato has natural resistance to insect pests, intensive agricultural practices created an artificial environment that favors insect pest proliferation. Today, a tremendous increase in the use of chemicals to control insect pests has created a strong pressure for the development of resistant insect populations and reduced insect predators' populations. Because of this, current efforts to develop insect-resistant crops are following the theme of transforming plants with a single gene encoding insecticidal enzymes or toxins. The delta endotoxins of *Bacillus thuringiensis* are the most widely researched genes among the insecticidal enzymes or toxins (Barton and Miller 1994).

One of the key components of an integrated pest management program to control potato tuberworm is host plant resistance. At present, there is no potato material, produced from host plant resistance breeding that has appreciable levels of resistance to potato tuberworm (Lagnaoui et al. 2000). Combining natural host plant resistance and insect resistance conferred by a *Bacillus thuringiensis* gene in potato breeding against

potato tuberworm may increase the efficacy and stability of resistance (Coombs et al. 2002).

## GENETIC ENGINEERING

A potential solution for the improvement of cultivated potato for traits such as resistance to diseases, insect pests, herbicides, environmental stresses, yield and as well as various quality parameters is through the use of genetic engineering (Conner et al. 1994). One highly successful approach to engineering resistance has involved generating plants that synthesize antimicrobial or insecticidal products (Dempsey et al. 1998). In terms of genetically engineering potato for host plant resistance to insects, there are two classes of important toxin genes: those coding for toxins against the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera:Chrysomelidae), and those coding for toxins active against lepidopterous pests, such as potato tuberworm (PTW), *Phthorimaea operculella* (Zeller) (Lepidoptera:Gelechiidae) (Gould et al. 1994).

After the discovery of a successful transformation procedure using the vector *Agrobacterium tumefaciens*, potato has been used as a subject for genetic engineering experiments for the past 20 years. *Agrobacterium*-mediated gene transfer has been used as one of the most common transformation techniques in potato breeding (Conner et al. 1994). Introduction of transgenes into higher plants using *Agrobacterium*-mediated transformation method still remains as the predominant protocol used for potato (Belknap et al. 1994). This procedure offers the advantage of mobilizing defined regions of DNA into the nuclear genomes of the plant (Belknap et al. 1994).

***Bacillus thuringiensis***

Naturally occurring *Bt* strains possess genes which code for a number of distinct toxic proteins (δ-endotoxins) with different spectra of activity against insects (Gould et al.1994). Potato has been genetically transformed to express genes of various subspecies of *Bacillus thuringiensis* Berliner *(Bt)* encoding insecticidal protein (Head et al. 2002). For the past ten years, δ-endotoxins from different *Bacillus thuringiensis* (*Bt*) subspecies have been used to protect crops against insects (Dempsey et al. 1998). Transformation in potato using *Bt-cry* genes have advantages. The mode of action of these genes is highly specific or is highly host specific. A protein with specific toxicity towards coleoptera would not be toxic to other orders of insects, and *Bt* crystal proteins have not shown any toxicity towards humans, other mammals or birds (Coombs et al. 2002, Lavrik et al. 1995). Furthermore, transgenic plants deliver the *Bt* toxin gene with increased efficacy compared to foliar application, lowering application costs (Douches et al. 2001). At present, over 10 million hectares are planted to *Bt* crops globally, mainly with plants expressing toxins against lepidopteran pests, butterflies and moths (Ferry et al. 2004)

*Bt* produces different crystal (Cry) insecticidal proteins during sporulation, which provide protection against a wide range of insect pests (Jenkins and Dean 2001). The crystal protein, when ingested, is solubilized in the insect's midgut and releases δ-endotoxins upon activation by the insect's midgut proteases. The activated toxins bind to the midgut epithelium of the insect causing disruption in membrane integrity leading to rapid death (Gill et al. 1992). The insecticidal crystal protein produced by *Bacillus thuringiensis* var. *kurstaki* is a toxin specific for Lepidoptera larvae (Chan et al. 1996). Among the first genes that have been used in genetic transformation of plants for improved insect resistance were genes encoding *Bt* crystal proteins (Theunis et al. 1998).

*Bt* toxin genes have been cloned, sequenced and codon-modified to increase expression level in plants and are now being used in various crop species (Mohammed et al. 2000). Potatoes can be transformed using *Agrobacterium tumefaciens* Ti plasmid-mediated genetic transformation (Douches et. al. 1998). When inserted into crop plants, the efficacy of wild type *Bt* genes is less than that of a codon-modified *Bt-cry1* and *Bt-cry3* genes (Douches et. al. 1998). Transformation with wild type *cry 1ac* Bt toxin gene specific for Lepidoptera have produced potatoes with low levels of Bt-expression causing from 20-60% insect mortality in detached leaf bioassays (Mohammed et. al. 2000). The use of a codon-modified *Bt* gene could provide increased receptor binding and toxicity, broadening the spectrum of activity against target pests, and reducing effects on non-target insects (Jerkins and Dean 2001).

**Objectives of the study**

1) transform three potato lines which differ in natural host plant resistance (susceptible, glandular trichomes, increased glycoalkaloids) with a codon-modified *Bt-cry1ac* gene;

2) conduct laboratory bioassays to test the potato tuberworm response on *Bt- cry 1ac* transgenic lines and;

3) conduct molecular characterization of the *Bt-cry 1ac* transgenic lines to verify gene insertion, to quantify the amount of protein and to determine the number of inserted copies of the gene.

## MATERIALS AND METHODS

### VECTOR CONSTRUCT

pSP 73 *cry1a(c)* improved [from John Kemp, New Mexico State University] was digested with *XhoI* and T4 DNA Polymerase treated to create a blunt end. It was then digested with *BamHI* and the 1.76 Kb fragment was isolated. This fragment was ligated to the 11 Kb fragment of pBI121 which was cut with *BamHI* and *EcoICRI*. The resulting plasmid is the pSPUD15 (Fig.1). The plasmid includes the following: RB, T-DNA right border; NOS-T; *cry1a(c), Bt cry1a(c)* insecticidal protein gene; CaMV 35S, promoter; NOS-T; *nptII*, neomycin phosphotransferase gene confers resistance to kanamycin; NOS-Pro; LB, T-DNA left border.

### PLANT MATERIAL

Three potato varieties were used for *Agrobacterium*-mediated transformation experiment: Spunta, ND5873-15, and NY123. Spunta is a long, white fleshed, tablestock cultivar that was bred in Netherlands. It is grown widely in subtropical regions such as North Africa and South America (Douches et. al 2002). NY123 is a breeding line from Cornell University that has higher densities of type A glandular trichomes. ND5873-15 is a breeding line from North Dakota State University (NDSU) that has higher levels of an undefined glycoalkaloid in the foliage.

### TRANSFORMATION PROTOCOL

*A. tumefacians*-mediated transformation as described by Douches et al. (1998) and Step I and Step II regeneration media (Yadav and Sticklen 1995) were used in the

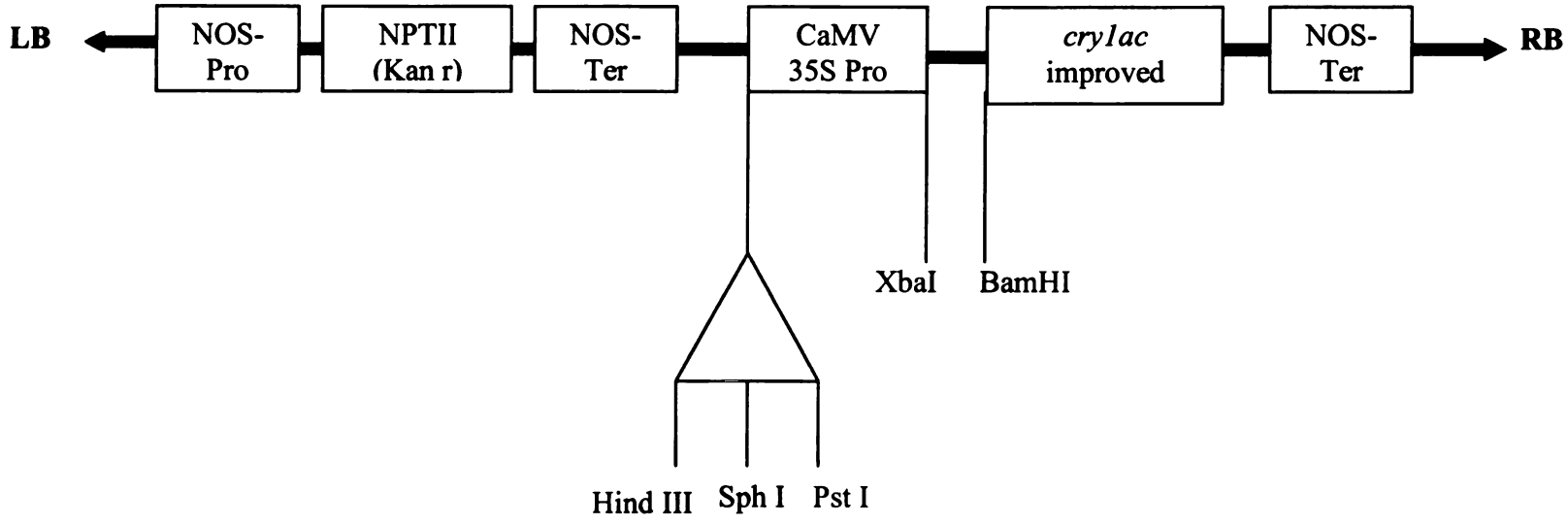


**Figure 1. Schematic diagram of pSPUD 15 vector construct. RB, T-DNA right border; NOS-T; c*ry1ac*, *Bt cry1ac* insecticidal protein gene; CaMV 35S, promoter; NOS-T; *NPTII*, neomycin phosphotransferase gene confers resistance to kanamycin; NOS-Pro; LB, T-DNA left border.**

generation of the transgenic lines of Spunta, NY123 and ND5873-15. Potato lines were prepared for transformation by removing the tip and the petiole ends from tissue culture plantlet leaves. The 25-30 leaves were then placed top-surface down on the solid Step I media (MS salts, 3 % sucrose, 0.9 $mgl^{-1}$ thiamine-HCl, 0.5 $mgl^{-1}$ trans-zeatin riboside, 2 $mgl^{-1}$ 2,4-D, 7 $gl^{-1}$ Bactoagar, pH 5.7) and precultured for 2-4 d (Yadav and Sticklen 1995). After 2 d, the precultured potato leaves were soaked in the log-phase *A. tumefaciens* suspension for 5-10 min. The soaked leaves were blotted briefly on sterile paper towels to remove excess liquid and then they were transferred to fresh solidified Step I media for 4 d. After 4 d, the leaves were washed using of 30 ml of sterile double distilled water with 30µl Timentin. The leaves were blotted briefly on sterile paper towels to remove excess liquid and then they were transferred to solid Step II media containing 50 $mgl^{-1}$ kanamycin and 200 mg $l^{-1}$ Timentin (Smith Kline Beecham, Philadelphia, PA) in 10 x 100 mm Petri dishes. Leaves were transferred every 7-10 days to fresh solidified Step II media.

Once the callus nodules produced 5-7 mm long shoots, shoots were excised and placed in the rooting media (modified MS media with the addition of 50 $mgl^{-1}$ kanamycin and 200 mg $l^{-1}$ Timentin) in 25 x 150 mm culture tubes. One shoot per callus was removed and transferred to the kanamycin rooting media. As soon as the shoot was removed, the callus was then excised to prevent it from producing other shoots. This was done to prevent production of similar transgenic lines. Timentin was added to prevent the over-growth of the *A. tumefaciens* on the media.

Rooted shoots were grown in the tubes with rooting media and were propagated in Magenta G5 vessels (5 plants/vessel). Four to five-week-old plants were transferred to

seedling trays (50/tray) in the greenhouse and transplanted to 10 cm diameter pots in the greenhouse for tissue collection, bioassays and molecular characterization.

## POTATO TUBERWORM REARING

A potato tuberworm population from South Africa has been maintained at the Department of Entomology Michigan State University since 2004. The culture was maintained at 25°C on potato tubers following the rearing method described by Mohammed et al. (2000). Eggs laid on the no.1 Whatman filter paper (Whatman, Hillsboro, OR) were placed on top of sliced potato tubers in the Petri dish. Newly hatched larvae (1 day old) were used in the detached-leaf bioassay.

## DETACHED-LEAF BIOASSAY

The mortality of the potato tuberworm feeding on Bt-*cry1ac* transgenic lines was tested using a detached leaf bioassay (Westedt et al. 1998). Fresh leaf was collected and the leaf petiole was inserted in a sponge fitted in a glass vial full of water. The vial was sealed using Parafilm to prevent water leakage. The leaf and the vial were placed in a Petri dish (25 x 150 mm) with filter paper. Ten neonate larvae were placed on the surface of each leaf. Each Petri dish was considered as a replication. Four replications per transgenic line were used and the Petri dishes were arranged in a completely randomized design. Counting of the live larvae and observation of their mining, growth and general health was done under a dissecting microscope. Evaluation of larval mortality was made and recorded 3 d after infestation.

TUBER BIOASSAY

Antibiosis was tested in the laboratory by placing tubers and potato tuberworm larvae together in a closed containers (no-choice test) with vermiculite. Greenhouse tubers were harvested from Spunta, NY123 and ND5873-15 lines. Each tuber was placed inside a closed container with vermiculite and was exposed to 10 neonate larvae. Each container was considered as a replication. Four replications per transgenic line were used and the containers were arranged in a completely randomized design. Tests were carried out in an insect mass rearing room under controlled temperature and humidity (25 ± 2°C and 70 ± 5% relative humidity). After 21 d, the number of pupae developed was recorded and the overall mortality were expressed in percentage.

**MOLECULAR CHARACTERIZATION**

POLYMERASE CHAIN REACTION (PCR)

Presence of the *cry1ac* gene in the transgenic potato lines was determined using PCR. Isolation of total genomic DNA from greenhouse plants was done using the DNeasy Plant Mini method (Qiagen, California). Each putative transgenic potato plant DNA was used as a template. The *cry1ac* gene specific primer were used in the PCR. The primers used for the *cry1Ac* gene were 5' CAT GGC TAT CGA GAC CGG TTA CAC TCC 3' and 5' CTG TCT ATG ATC ACA CCT GCA GTT CC 3'. The expected band size was 1.8 Kb.

The PCR components for 20 µl reactions included 2µl of 10X buffer (Promega, Wisconsin), 2µl of 5.0 mM dNTP mixture, 0.6µl of 25mM $MgCl_2$ (Promega, Wisconsin), 0.17µl of 3ODU & 0.13µl of 3.8ODUof CryIAc primers, 3µl of templates DNA (4-

104ng/ul), 2.5 U Taq DNA polymerase (Promega, Wisconsin), and water to a total volume of 20µl.

The PCR amplification conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min 30 sec, and

primer extension at 72 °C for 1min 30 sec, and a final extension at 72 °C for 4 min. The reactions were held at 4 °C before the analysis. Reaction products were then electrophoresed on a 1 % (w/v) agarose gel (Boeringer Mannheim, Indianapolis, IN) containing ethidium bromide at 0.5 mg $ml^{-1}$ in 1x Tris-acetate EDTA buffer (pH 8.0) at 80 mV for 1 h 30 min and viewed under ultraviolet light (254 nm).

SOUTHERN ANALYSIS

*Plant DNA extraction*

Total plant genomic DNA was extracted from the fresh leaf tissue of greenhouse-grown tissue culture transplants using the CTAB extraction protocol (Saghai-Maroof et al. 1984), modified by adding 2 % beta-mercaptoethanol to the extraction buffer. The DNA was quantified using a mini-flourometer following the manufacturer's instructions (Amersham Pharmacia Biotech, San Francisco CA, Hoeffer Scientific, San Fernando, CA, model Hoefer DyNA® & Quant 200®).

*DNA Probe Labelling*

The plasmid *cry1ac* gene was isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, Wisconsin).

Polymerase Chain Reaction (PCR) was used to amplify the *cry1ac* gene in the plasmid DNA. The PCR components for 20 µl reactions included 10 µl of 2X Red Taq

Ready mix (Sigma, Saint Louis, Missouri), 1 µl of 5µM & 1µl of 5µM of *cry1Ac* forward & reverse primers, 1µl of template plasmid DNA 25 ng/ul, and water to a total volume of 20µl.

The bands in the gel were cut off and were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, Ca.). After gel purification, the plasmid DNA yield was quantified via spectrophotometer. The highest concentration of the DNA plasmid yield was 50ng/ml and was used for the probe.

The purified plasmid DNA was used as the probe and was labelled using the DIG High Prime DNA Labeling and Detection Starter Kit II following the manufacturer's instructions (Roche, Mannheim, Germany 2004).

*DNA Digestion*

DNA was digested using Xba I (Roche, Penzberg, Germany). The digestion reaction components included 3 µl of 10x buffer, 6 µl of 10 U/ µl Xba I, 25 µg of genomic DNA and 1µg of plasmid DNA, and water to a total volume of 30µl. After 3 h of digestion, the digested DNA fragments were then electrophoresed in a 1.2 % (w/v) agarose gel (Boeringer Mannheim, Indianapolis, IN).

*Blot Transfer*

The fragments produced in the gel were transferred to a nylon membrane (Hybond N+, Amersham Life Sciences, Buckinghamshire, England) via Southern blotting techniques following the manufacturer's procedures.

*Hybridization and detection of gene insertion*

Hybridization was conducted overnight at 42°C in a fresh solution containing approximately 25 ng $ml^{-1}$ DIG-labeled DNA probe. Chemiluminescent detection was

conducted following the manufacturer's procedures using 75 $mUml^{-1}$ anti-digoxigenin alkaline-phosphatase conjugate and 1ml CSPD ready-to-use substrate (Roche, Manheim, Germany). The membrane was then exposed to X-ray film (Hyperfilm MP, Amersham Life Sciences, Buckinghamshire, England) for 15-30 min and developed.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Pathoscreen kit from Agdia (Elkhart, IN) was used to detect the Bt-CryIAc protein expressed in each transgenic plant. Fresh leaves were harvested from the greenhouse. Collected leaf tissues were put in sample extraction bags. Fresh leaf tissues (100 mg) was ground using an Agdia homogenizer attached to a 10" drill mess (Agdia, Elkhart, IN) and extracts were diluted with PBST buffer to a ratio of 1:10 (w/v). The enzyme conjugate (100 µl) was added per well and then 100 µl of each prepared sample was dispensed in each test well of the ELISA plate. Also, 100 µl of positive and negative control were dispensed in the appropriate testwells. The plate was sealed with parafilm, to prevent moisture loss, and was incubated for 2 hours. When the incubation period was completed, the plate was washed with 1x PBST buffer 7 times. After washing, the wells were soaked with 1x PBST buffer and allowed to sit for 1 min. After the soaking, 100 µl of the TMB substrate solution was added in each well. The plate was set aside for 15 min for the color to develop. Those wells that changed their color from colorless to blue were scored positive and those wells that did not change their color or changed their color to light blue were scored negative for presence of protein. After the color was developed, 50 µl of 3M of sulfuric acid was added in each well. The optical density was then measured using the Wallac $Victor_{Tm}^{2}$ V plate reader (Perkin Elmer Life Sciences, Downers Grove IL) at 450nm.

A standard curve was constructed from the controls and was used to compute the Bt-*cry1Ac* protein expression in ng/ml. The amount of protein per milligram of fresh leaf tissue was computed using the formula below:

$$\text{Protein (ng/mg)} = \text{Protein conc. (ng/ml)} \times \frac{1\text{ml}}{100\text{ mg of fresh leaf tissue}}$$

STATISTICAL ANALYSIS

Percentage mortality data for the bioassays were subjected to arcsine (degrees[asin(sqrt(DEF/100))]) transformations before analysis of variance. Means were compared using the Least Significant Difference (LSD) in the general linear model procedure ($LSD\alpha_{=0.05}$) of SAS (SAS Inst., Inc., 2001). The transformed mortality was converted to percentage mortality ([(sin(radians(ARCSIN)))^2]*100).

# RESULTS

## POTATO TRANSFORMATION WITH *cry1ac* GENE

It took 4-7 weeks for NY123 to produce shoots from the leaf explants. A total of 23 shoots from NY123 were removed and transferred to individual tubes containing kanamycin (50mg/l) media (Table 1). From the 23 shoots, 3 rooted and grew in the kanamycin rooting media. Out of the 3 rooted, none tested PCR positive for *cry1ac* gene. Line NY123 was the easiest to regenerate shoots but was the most resistant to transformation.

A total of 32 putative transgenic potato shoots were produced from Spunta leaf explants. Out of the 32 shoots, 12 rooted in the kanamycin media. These 12 shoots that rooted were then regenerated into plants. Eleven of 12 rooted plants tested positive via PCR for having the *cry1ac* gene. Spunta was slower to regenerate shoots compared to NY123. On the average, it took 5-8 weeks for Spunta to produce shoots.

Line ND5873-15 was the slowest in shoot regeneration, it required 8-10 weeks for ND5873-15 to produce shoots. A total of 34 putative transgenic potato shoots were produced from the leaf explants and were transferred to individual tubes containing kanamycin rooting media. Out of the 34 shoots, 12 rooted and were regenerated into plants. The eight of 12 rooted shoots were confirmed PCR positive for the presence of *cry1ac* gene.

## MOLECULAR CHARACTERIZATION

Presence of *cry1ac* gene in almost all (91.7%) of the rooted Spunta shoots and 66.7% of ND5873-15 rooted shoots was confirmed positive by PCR (Figure 2). All the

Table 1. Results of kanamycin rooting assay and *cry1ac* PCR amplification of putative NY123, Spunta and ND5873-15 transgenics.

| Potato Line | Resistance Factor | No. of Shoots | No. of rooted Shoots[a] | No. of *cry1ac* PCR positive lines |
|---|---|---|---|---|
| Spunta | None | 32 | 12 | 11 |
| ND5873-15 | Glycoalkaloid | 34 | 12 | 8 |
| NY123 | Glandular trichome | 23 | 3 | 0 |

[a] The kanamycin concentration used in the rooting media was 50 mg per liter.

Figure 2. PCR analysis of putative *cry1ac* transgenic lines of Spunta and ND5873-15.

*cry1ac* transgenic lines that tested PCR positive from Spunta and ND5873-15 were subjected to ELISA test for protein expression and detached leaf bioassays. Southern blotting and ELISA tests were used as the confirmatory analysis for the *cry1ac* gene insertion and *Cry1ac* protein expression. The four high performing transgenic lines from ND5873-15 and transgenic lines from Spunta identified from the detached-leaf bioassay were further characterized using the Southern blotting analysis.

*Cry1ac* protein expressions in Spunta and ND5873-15 transgenics varied between 0 to 0.58 ng/mg of fresh leaf tissue (Table 2). In general, it was observed that the levels of protein expression in the ND5873-15 *cry1ac* transgenics were higher than levels in Spunta transgenics. The average protein expression from ND5873-15 transgenics was 0.17 ng/mg of fresh leaf tissue, while it was 0.03 ng/mg of fresh leaf tissue in *cry1ac* Spunta transgenics. The highest expression of *Bt-Cry1ac* protein was observed in ND15.11 and ND15.10. They contained 0.58 ng and 0.32 ng of *Bt-Cry1ac* protein/mg of fresh leaf tissue, respectively. On the other hand, no protein expression was observed from four Spunta transgenic lines Sp15.04, Sp15.08, Sp15.10 and Sp15.13. The controls, Spunta and ND5873-15, tested negative for the *Bt-Cry1ac* protein expression.

Results of Southern blotting showed that the number of inserted *cry1ac* genes for both Spunta and ND5873-15 transgenics varied between one to two copies (Figure 3). All the ND5873-15 *cry1ac* transgenics had single insertion except for ND15.10 which had two copies inserted. Similarly, all the Spunta *cry1ac* transgenics had a single gene insertion except for Spunta15.07 line which has two copies inserted. Southern blot analysis showed duplicate lines both in ND5873-15 (ND15.05 and ND15.08) and Spunta transgenic lines (Sp15.01 and Sp15.05). Duplication was also supported by the mortality data and the protein expression data. The same mortality (55%) was observed in both

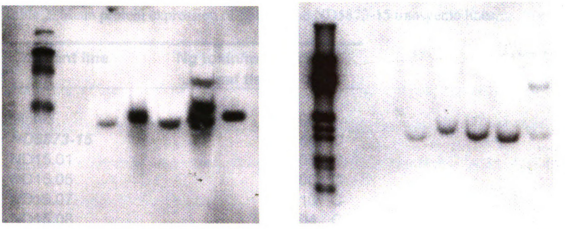


Fig. 3. Southern analysis of ND5873-15 and Spunta *cry1ac* transgenic lines. DNA was digested using XbaI and probed with *cry1Ac* gene.

Table 2. Mean protein expression of Spunta and ND5873-15 transgenic lines.

| Plant line | Ng toxin/mg of fresh leaf tissue* | |
|---|---|---|
| Blank | 0.00 | |
| ***ND5873-15*** | ***0.00*** | ***C*** |
| ND15.01 | 0.05 | C |
| ND15.05 | 0.06 | C |
| ND15.07 | 0.10 | C |
| ND15.08 | 0.04 | C |
| ND15.10 | 0.32 | B |
| ND15.11 | 0.58 | A |
| ND15.12 | 0.01 | C |
| ND15.13 | 0.01 | C |
| ***Spunta*** | ***0.000*** | ***C*** |
| Sp15.01 | 0.02 | C |
| Sp15.02 | 0.08 | C |
| Sp15.03 | 0.03 | C |
| SP15.04 | 0.00 | C |
| Sp15.05 | 0.06 | C |
| Sp15.06 | 0.04 | C |
| Sp15.07 | 0.05 | C |
| Sp15.08 | 0.00 | C |
| Sp15.10 | 0.00 | C |
| Sp15.11 | 0.01 | C |
| Sp15.13 | 0.00 | C |

* Means with the same letter designation are not significantly different as determined by Fisher's Protected LSD ($\alpha$=0.05).

ND15.05 and ND15.08. Similarly, the protein expression for both lines was not significantly different from each other. They contained 0.06 and 0.04 ng of *Bt-Cry1ac* protein/mg of fresh leaf tissue, respectively. On the other hand, mortality in Sp15.01 (55%) was not significantly different from mortality in Sp15.05 (56%). Also, no significant difference was observed between the protein expression in Sp15.01 (0.02 ng/mg) and Sp15.05 (0.06 ng/mg).

DETACHED LEAF BIOASSAY

The analysis of variance for the percent mortality of potato tuberworm in Spunta transgenic lines and control showed highly significant differences among lines ($P<0.0001$). Similarly, significant differences among lines ($P = 0.02$) were observed between ND5873-15 transgenic lines and control.

Percent potato tuberworm mortality was moderately low (22 %-68 %) for almost all the *cry1ac* transgenic lines except for the two lines from ND5873-15 namely ND15.10 and ND15.11 (Figures 4 & 5). These two lines caused 73 % mortality. In general, the mortality in the Spunta control was significantly lower than mortality in the ND5873-15 control and mortality from Spunta transgenics was lower than mortality with ND5873-15 transgenics. The average mortality for Spunta *cry1ac* transgenic lines was 49% and 57% for ND5873-15 *cry1ac* transgenic lines. Potato tuberworm mortality from all the Spunta *cry1ac* transgenic lines (22% - 58 %) was significantly higher than mortality in Spunta (3 %). Potato tuberworm mortality for six ND5873-15 transgenics: ND15.10, ND15.11, ND15.07, ND15.08, ND15.05 and ND15.01 (36 %-73 %), were significantly higher than mortality with the control ND5873-15 (22 %). Mortality with ND15.12 and ND15.13 lines was not significantly different from mortality with the control ND 5873-15.

ND15.10 and ND15.11, produced significantly higher potato tuberworm mortality compared with mortality with many of the other ND5873-15 transgenic lines or the control ND5873-15. There was a significant correlation between mortality and protein expression for the ND lines ($r^2 = 0.50$, $P = 0.03$) and for the Spunta lines ($r^2 = 0.40$, $P = 0.02$).

ND15.10 and ND15.11 showed the highest level of control against potato tuberworm. These two lines were selected and included for further detached-leaf bioassay evaluation. Unlike the initial detached-leaf bioassay where the evaluation was done after 3 d, evaluation for the advanced detached-leaf bioassay was after 5 d. Analysis of variance for the percent mortality of potato tuberworm in the 5-day detached-leaf bioassay for ND5873-15 control and ND5873-15 *cry1ac* transgenics showed highly significant differences among lines ($P<0.0001$) (Figure 6). Potato tuberworm mortality in both ND15.10 (88%) and ND15.11 (97%) lines was significantly higher than mortality in the control ND5873-15 (17%). Potato tuberworm mortality with ND15.11 was significantly higher than mortality with ND15.10 or the control ND5873-15. Both lines ND15.10 and ND15.11 expressed the highest levels of *Cry1ac* protein and caused the highest mortality in the detached-leaf bioassay compared with the other Spunta and ND5873-15 *cry1ac* transgenic lines.

## TUBER BIOASSAY

Analysis of variance for the percent mortality of potato tuberworm in the tuber bioassay for ND5873-15 control and ND5873-15 *cry1ac* transgenics showed highly significant differences among lines ($P<0.0001$) (Figure 7). Potato tuberworm mortality in both ND15.10 (97 %) and ND15.11 (99 %) was significantly higher compared with

mortality in the ND 5873-15 control (22 %). Potato tuberworm mortality in ND15.10 was not significantly different with mortality in ND 15.11. Significantly higher levels of resistance was observed in both ND15.10 & ND15.11 compared to ND5873-15 in the tuber & detached-leaf bioassays.

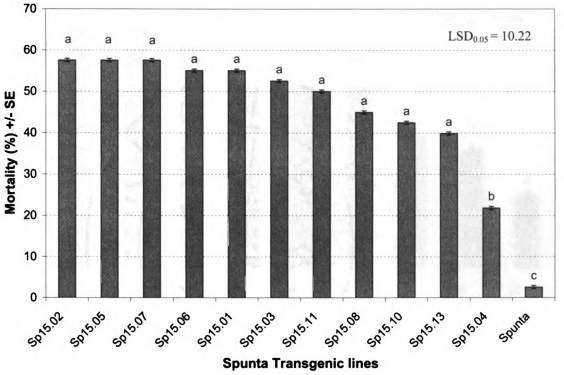


Fig 4. Mean percent mortality (detached-leaf bioassay) of first instar of potato tuberworm on Spunta and Spunta transgenic lines. Means with the same letter designation are not significantly different as determined by Fisher's Protected LSD ($\alpha$ = 0.05).

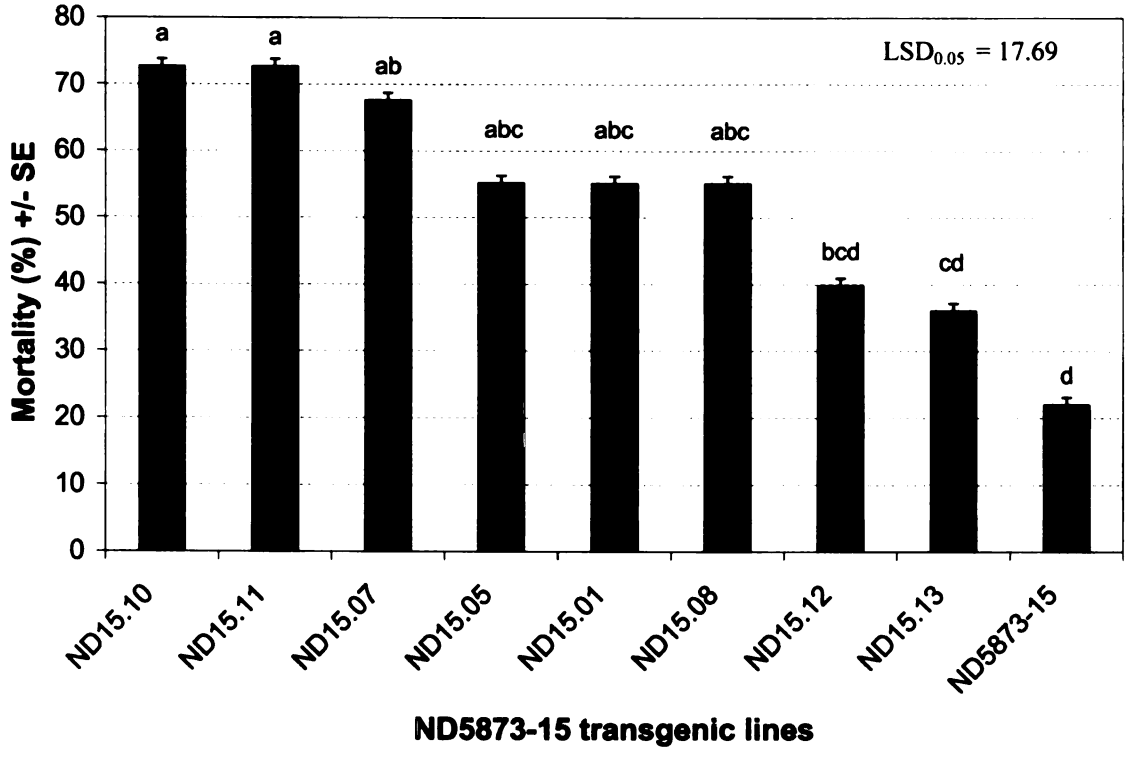


Fig. 5. Mean percent mortality (detached-leaf bioassay) of first instar of potato tuberworm on the ND5873-15 and ND5873-15 transgenics. Means with the same letter designation are not significantly different as determined by Fisher's Protected LSD ($\alpha$ = 0.05).

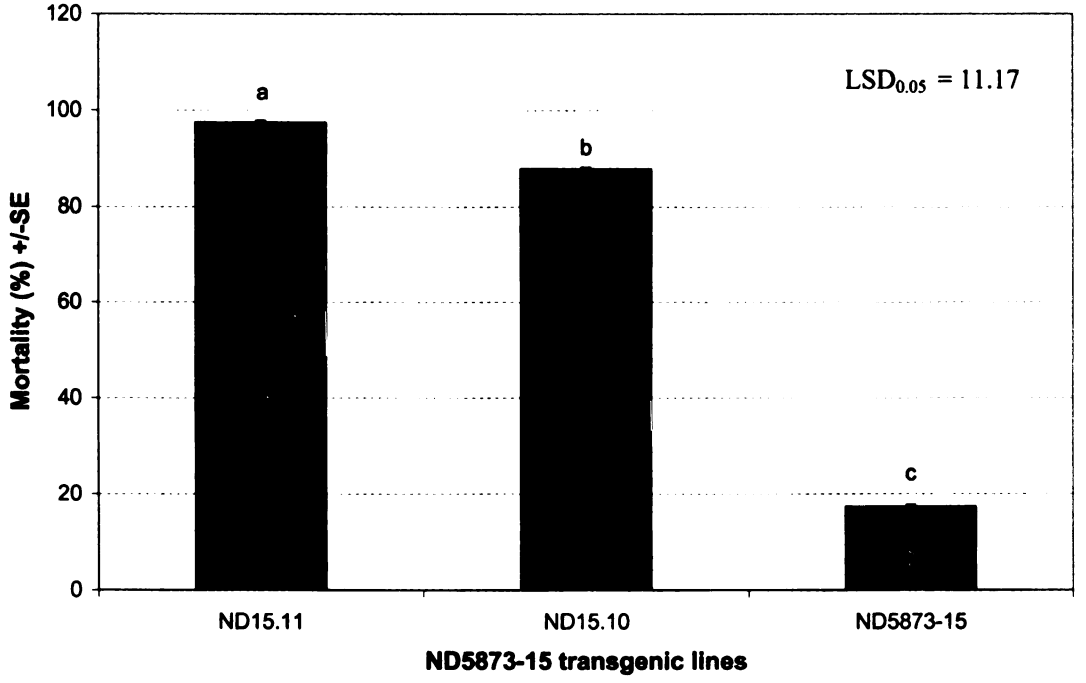


Fig. 6. Mean percent mortality (5-day detached-leaf bioassay) of first instar of potato tuberworm on the control ND5873-15, ND15.10 and ND15.11. Means with the same letter designation are not significantly different as determined by Fisher's Protected LSD ($\alpha = 0.05$).

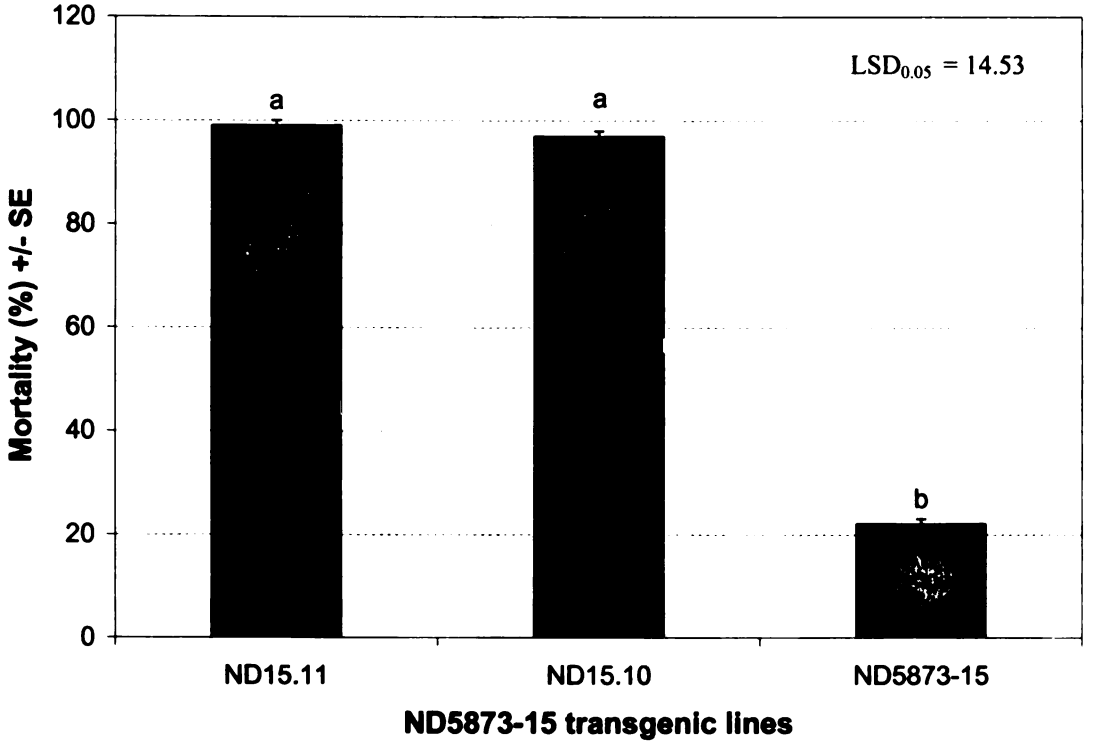


Fig. 7. Mean percent mortality (tuber bioassay) of first instar of potato tuberworm on the ND5873-15 and ND5873-15 transgenic lines. Means with the same letter designation are not significantly different as determined by Fisher's Protected LSD ($\alpha = 0.05$) .

## DISCUSSION

The potato transformation of *cry1ac* gene using the protocol in our laboratory was effective in regenerating shoots of transgenic potato lines in both Spunta and ND5873-15 lines. The stringency of selection using the *nptII* gene was effective for Spunta and ND5873-15 and made the number of test samples manageable. Although the production of transgenic shoots from both Spunta and ND5873-15 were successful, NY123 was found to be resistant to transformation. NY123 was obviously difficult to transform since the frequency of shoots that rooted in the kanamycin media was very low (13 %). Furthermore, none from the NY123 shoots tested positive for *cry1ac* gene via PCR. Differences in the ease of producing transgenic lines between cultivars were also observed by Felcher et al. (2003). In their study, they recovered a significantly higher number of transformed Spunta and Atlantic glucose oxidase transgenic lines compared to Libertas (which gave them few transgenic lines). Similarly, Coombs et al. (2002) transformed three different potato lines, Yukon Gold, USDA8380-1 and NYL235-4 with *Bt-cry3a*, and observed that NYL235-4 produced significantly lower number of rooted shoots in the kanamycin medium compared with the other two lines. NY123 used in our study, is a progeny of NYL235-4 (W. De Jong, pers. communication). These differences in the successful regeneration of transgenic shoots between cultivars/lines suggest that there is genetic variation for regeneration and transformation efficiency (Felcher et al. 2003).

Southern blot analysis showed that the number of insertion events in the Spunta and ND5873-15 *cry1ac* transgenic lines were one to two copies. Similar results were observed by Davidson et al. (2002). They observed that all five of the highest performing

transgenic lines had either one or two copies of *cry1ac9* gene. Douches et al. (1998) observed copy numbers of *Bt-cry5* gene ranging from one to three. Copy numbers ranging from one to seven of glucose oxidase gene in transgenic potato lines were observed from the study conducted by Felcher et al. (2003). Similarly, Beuning et al. (2001) also observed one to seven copies of *cry1ac9* gene inserted in tobacco transgenic lines.

Lower level of protein expression was observed in Sp15.7 as compared with expression in ND15.10 and ND15.11 Although, Sp15.07 had two inserted *cry1ac* genes it still gave lower level of protein expression compared to ND15.10 (which has also two inserted cry1ac genes) and ND15.11 (which has only one inserted cry1ac gene). In this study, the number of inserted copies of *cry1ac* gene in the plant genome does not correlate with *Cry1ac* protein expression. Similarly, Felcher et al. (2003) observed that there was no correlation between copy number and gene silencing. Different results were observed by Davidson et al. (2002), higher protein expression was produced from the transgenic potato lines with two inserted copies of cry genes as compared with transgenic potato lines with one inserted gene copy.

The mortality data showed that the highest resistance to potato tuber worm was observed only from ND15.11 and ND15.10 lines. The high mortality observed from both lines did correspond to their high *Cry1ac* protein expression. The ability to achieve high protein expression from both lines may be because synthetic codon-modified *cry* genes like *cry1ac*, give expression that is significantly higher compared to native *cry* genes encoding protoxins (Kuvshinov et al. 2001). Native *cry* genes are expressed poorly in the transgenic plants, thus the resistance to insect pests is minimal (Beuning et al. 2001). Protein expressions from *cry* gene constructs are about 0.1-0.3% of soluble protein

corresponding to about 1 μg of toxin protein per 1g of fresh leaf tissue (Kuvshinov et al. 2001). In a study conducted by Perlak et al. (1990) as cited by Babu et al. (2003), transformed cotton with codon-modified *cry1ab* and *cry1ac* gene and observed protein expression of 0.05-0.1% of the total soluble protein. Variation in the level of resistance to potato tuberworm was observed from the independently derived Spunta and ND5873-15 *cry1ac* transgenic lines. The variation in the level of transgene expression is common among the population of plants that are independently transformed with the same transgene. This unpredictable expression is usually attributed to position effects resulting from the random integration of transgenes into different sites of plant genomes (Davidson et al. 2002, Conner and Christey 1994).

The levels of protein expressions obtained from this study were higher compared to the protein expression levels observed by Davidson et al. (2002). They observed that the amount of *Cry1ac9* protein in all the transgenic potato lines they tested was less than 60 ng/g of fresh leaf tissue. In the study conducted by Kuvshinov et al. (2001) they observed comparable amount of *Cry9Aa* protein expression (300 ng/g) in potato plants and higher *Cry9Aa* protein expression (1.4 μg/g of leaf material) in tobacco plant.

There was a significant correlation between mortality in the feeding assay and the ELISA data for protein expression for both ND5873-15 and Spunta lines. Variability in this correlation lies in the variable responses of the insect larvae and also in the variability inherent in the ELISA determination of protein expression. In a feeding assay, larvae quit feeding as soon as they became intoxicated. Larvae feeding on potato foliage with low concentrations of *Bt* may have fed longer and consumed more foliage, compared to the amount consumed by larvae feeding on foliage with high concentrations of *Bt*. Thus, the dose received (amount of consumed foliage X *Cry1ac* protein

concentration) and the resulting mortality may not have been directly proportional to Bt concentration. Mortality in the transgenic lines is not only dependent on the amount of protein expressed in the leaves but is also influenced by the total amount of *Cry1ac* protein ingested by the potato tuberworm.

The resistance provided by the integration of *cry1ac* gene in this study, up to 97 % potato tuberworm mortality, was similar to the resistance provided by the integration of *Bt-cry5* gene in the study conducted by Westedt et al. (1998). They observed that potato transformation with a codon-modified *Bt-cry5* gene provided resistance against potato tuberworm of up to 96% mortality. In the study conducted by Mohammed et al. (2000), they observed higher potato tuberworm mortality of up to 100 % in the same *Bt-cry5* Spunta transgenic lines. Similarly, 100 % potato tuberworm mortality after 10 d was observed with both *cry1ac* and *cry1ab* potato transgenic lines (Davidson et al. 2002). Jansens et al. (1995) observed protein content of 3-118 ng toxin/mg total protein in the leaves in the Yesmina and Kennebee *cry1ab* transgenic lines causing 40 %-100 % potato tuberworm mortality.

Higher mortality observed in the ND5873-15 control compared with mortality in the Spunta control is probably the result of natural insect resistance factors (glycoalkaloids) incorporated into the ND5873-15 (Lachman et al. 2001). The low mortality in the ND5873-15 (22 %) in spite of the fact that it has natural resistance (glycoalkaloids) may be attributed to the level of glycoalkaloids content in the leaves used in the detached-leaf bioassay. The glycoalkaloids concentration in potato plants can be highly affected by the temperature, light intensity and day length. Glycoalkaloids are present at higher concentration in the aerial (leaves, stems and sprouts) part of the potato plant and are normally present in lower concentration in the tubers (Lachman et al. 2001).

Similarly, Lafta and Lorenzen (2000) observed a significant increase in foliar glycoalkaloids when plants were grown at higher temperature (32/27 °C).

Higher mortality observed in the 5-day detached-leaf bioassay with ND15.10 & ND15.11 compared with the mortality on the initial detached-leaf bioassay can be attributed to the additional 2 d. Longer exposure of the potato tuberworm larvae to the protein/toxins could lead to higher toxin ingestion and higher mortality. Running the detached-leaf bioassay for 7-10 d is highly recommended to ensure longer exposure of potato tuberworm to the toxin. Results from the study conducted by Davidson et al. (2002) suggests that running the detached-leaf bioassay for up to 10 days could give higher potato tuberworm mortality of up to 100%. For the *Cry1ac* protein to have maximum effect in the Spunta and ND5873-15 transgenic lines, it may require exposure time of up to 7-10 d.

The production of more independently selected transgenic lines will allow recovery of several lines with high transgene expression, as well as phenotypically normal apprearance and yield performance (Meiyalaghan et al. 2004, Conner and Christey 1994). Field and storage trials are also important and necessary tests in the identification of high performing transgenic potato lines with good transgene expression, phenotypic appearance and yield equal to that of the commercial cultivars in the market.

Further study on the inheritance of *cry1ac* gene in their progeny is also interesting since (Babu et al. 2003) it is common that the genes showing expression in transgenic plants are stably inherited into the progeny without detrimental effects on the recipient plant.

The results of this study showed that combining natural host plant resistance and engineered resistance with the *Bacillus thuringiensis-cry1ac* gene in potato plant was

effective in conferring increase resistance to potato tuberworm in detached-leaf bioassays. The *cry1ac* gene can be another source of resistance which can be pyramided with other genes for host plant resistance to potato tuberworm in potato.

# APPENDIX A

Table 3. DNA concentrations used in the PCR reaction.

| Lines | Concentration (ng/μl) |
|---|---|
| Spunta | 18 |
| Sp15.01 | 11 |
| Sp15.02 | 18 |
| Sp15.03 | 30 |
| Sp15.04 | 30 |
| Sp15.05 | 31 |
| Sp15.06 | 76 |
| Sp15.07 | 47 |
| Sp15.08 | 49 |
| Sp15.10 | 23 |
| Sp15.11 | 104 |
| Sp15.13 | 59 |
| ND5873-15 | 4 |
| ND15.01 | 23 |
| ND15.05 | 15 |
| ND15.07 | 16 |
| ND15.08 | 33 |
| ND15.10 | 16 |
| ND15.11 | 12 |
| ND15.12 | 14 |
| ND15.13 | 18 |

## APPENDIX B

Table 4. Total glycoalkaloids contents of ND5873-15, ND5873-15 transgenic lines, Spunta and Spunta transgenic lines.

| Clones | TGA mg/g | Leptinines % | Leptines % | Solanine % | Chaconine % | Unknown % |
|---|---|---|---|---|---|---|
| Spunta | 5.33 | 0.00 | 0.00 | 7.25 | 29.19 | 63.56 |
| SP15.01 | 2.49 | 0.00 | 0.00 | 18.58 | 81.42 | 0.00 |
| SP15.02 | 11.61 | 0.00 | 0.00 | 3.99 | 22.90 | 73.11 |
| SP15.03 | 7.58 | 0.00 | 0.00 | 7.94 | 36.42 | 55.64 |
| SP15.04 | 8.04 | 0.00 | 0.00 | 7.82 | 40.36 | 51.82 |
| SP15.05 | 8.39 | 0.00 | 0.00 | 4.14 | 22.17 | 73.68 |
| SP15.06 | 7.74 | 0.00 | 0.00 | 7.06 | 37.49 | 55.45 |
| SP15.07 | 7.06 | 0.00 | 0.00 | 8.99 | 42.77 | 48.24 |
| SP15.08 | 13.14 | 0.00 | 0.00 | 3.99 | 19.19 | 76.82 |
| SP15.10 | 5.79 | 0.00 | 0.00 | 8.10 | 40.61 | 51.29 |
| SP15.11 | 4.82 | 0.00 | 0.00 | 10.42 | 38.26 | 51.32 |
| SP15.13 | 4.18 | 0.00 | 0.00 | 6.34 | 22.32 | 71.33 |
| ND5873.15 | 8.88 | 26.21 | 19.38 | 21.37 | 33.04 | 0.00 |
| ND15.01 | 24.25 | 26.87 | 33.58 | 17.43 | 22.12 | 0.00 |
| ND15.05 | 14.66 | 10.80 | 34.61 | 21.82 | 32.77 | 0.00 |
| ND15.07 | 29.89 | 8.32 | 40.83 | 20.99 | 29.86 | 0.00 |
| ND15.08 | 27.24 | 9.42 | 28.39 | 23.61 | 38.58 | 0.00 |
| ND15.10 | 18.21 | 14.36 | 34.11 | 17.99 | 33.54 | 0.00 |
| ND15.11 | 13.18 | 27.30 | 41.88 | 11.98 | 18.84 | 0.00 |
| ND15.12 | 28.63 | 12.87 | 44.30 | 17.09 | 25.74 | 0.00 |
| ND15.13 | 22.56 | 19.59 | 36.10 | 16.65 | 27.66 | 0.00 |

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