Potato Tuberworm (Lepidoptera: Gelichiidae) Resistance in Potato Lines with the Bacillus thuringiensis cry1Ac Gene and Natural Resistance

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Abstract. The potato tuberworm [Phthorimaea operculella (Zeller)] is one of the most destructive insect pests to potato (Solanum tuberosum L.) in tropical and subtropical regions, and it has recently become established in the Pacific Northwest of the United States. Combining natural resistance mechanisms with Bacillus thuringiensis (Bt) cry genes could be a potential solution to improve potato resistance to tuberworm. We have expressed Bt cry1Ac in two potato lines: Spunta, a susceptible potato line, and ND5873-15, a moderately resistant line with high foliar glycoalkaloids derived from Solanum chacoense. Putative transgenic lines of Spunta and ND5873-15 were developed using a vector construct pSPUD15 with the codon-modified Bt cry1Ac driven by the 35S CaMV promoter. Integration of Bt cry1Ac in Spunta and ND5873-15 transgenic lines was determined by PCR and Southern analysis. Protein expression in the transgenic lines (0–580 ng g⁻¹) was determined by ELISA. Plants expressing Bt cry1Ac were effective in controlling potato tuberworm first-instar larvae in the detached-leaf bioassays (up to 97% mortality) and in tuber bioassays (up to 99% mortality). Based on the assays conducted, the Bt cry1Ac Spunta lines were similar to the Bt cry1Ac ND5873-15 lines for potato tuberworm mortality. Constitutively expressed Bt cry1Ac would be a useful gene to use for host plant resistance to potato tuberworm.

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 United States. Estimated worldwide production is ≈330 million tons per year (FAOSTAT, 2004). In 2005, 440,000 ha were harvested in the United States alone, with a farmgate value of 2.9 billion USD (USDA-NASS, 2005). Potato is vulnerable to insect pests that affect both the foliage and the tuber (Flanders et al., 1999). Both pests and pathogens reduce yields and overall plant vigor. Due to the high economic value of the potato, pesticides are frequently applied to protect this crop.

Potato tuberworm, Phthorimaea operculella (Zeller), is an important and destructive insect pest to the potato worldwide (Flanders et al., 1999). The tuberworm is a common pest in tropical and subtropical regions, and it has recently become established in the Pacific Northwest of the United States. The potato tuberworm adult moth lays eggs on the underside of leaves or on exposed tubers. After hatching, the larvae molt three times, going through four instar stages, before pupating in leaf or soil debris. A complete life cycle can take place in as short as 4–5 weeks. Potato tuberworms cause damage both in the field and in storage by mining both the leaves and tubers, thereby reducing the quality of produce and increasing the risk of pathogen infection (Alvarez et al., 2005). Furthermore, the damage incitated by potato tuberworm tremendously reduces potato yield. In warmer climates, the quantity and quality losses in storage can be as high as 100% (Lagnaoui et al., 2000). Potato tuberworm is an increasing agricultural problem in the tropical and subtropical areas of the world due to its life cycle, feeding habits, and its ability to develop resistance to chemical insecticides (Alvarez et al., 2005).

Host plant resistance is a key component of an integrated pest-management program for potato tuberworm. Varying levels of resistance to insects occur naturally in crop plants and closely related species (Stoner, 1996). Potatoes contain glycoalkaloids that have long been known to possess antimicrobial properties and insect resistance (Sinden et al., 1980; Tingey, 1984). The two most common glycoalkaloids found in potatoes are α-solagine and α-solanine; together they comprise as much as 95% of the total glycoalkaloid present in the potato (Lachman et al., 2001). In this study, the North Dakota State University breeding line ND5873-15 was used for transformations because it has demonstrated resistance to the Colorado potato beetle in small plot field trials likely due to uncharacterized resistance factors and glycoalkaloids from the wild species Solanum chacoense (Coombs et al., 2005).

Genetic transformation offers considerable potential to improve insect and disease resistance to crops. Potato has been genetically transformed to express genes of various subspecies of Bacillus thuringiensis Berliner (Bt) (Douches and Grafius, 2005). The delta endotoxins of B. thuringiensis are the most widely researched genes among the insecticidal enzymes or toxins (Barton and Miller, 1993). The insecticidal crystal proteins produced by Bt var. kurstaki are toxins specific toward larvae of Lepidoptera (Chan et al., 1996). 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 (Douches and Grafius, 2005). The use of a codon-modified Bt gene can provide increased receptor binding and toxicity, broaden the spectrum of activity against target pests, and reduce effects on nontarget insects (Jenkins and Dean, 2001). Jansens et al. (1995) were able to achieve ≈100% potato tuberworm control with expressing the Bt cry1Ab6 in potato tubers. The Bt cry1Ac9 and cry1Aa2, using the potato light-inducible Lhca3 promoter, offered protection against potato tuberworm larval damage in foliage (Mejialaghan et al., 2006). Chakrabarti et al. (2006) expressed the Bt cry9Aa2 gene in tobacco chloroplasts and obtained resistance to potato tuberworm, but the high level of expression significantly delayed plant development.

Combining natural host plant resistance and engineered resistance conferred by a Bt gene in potato breeding against potato tuberworm may increase the efficacy and stability of resistance (Westedt et al., 1998). Combining different resistance factors assumes the insect is less likely to develop resistance to more than one toxin simultaneously; such strategies can delay resistance by orders of magnitude (Mani, 1985; Roush, 1998; Zhao et al., 2005).

The objectives of this research were to: 1) transform two potato lines that differ in natural host plant resistance (susceptible vs. increased glycoalkaloids) with a codon-modified Bt cry1Ac gene; 2) conduct molecular characterization of the Bt cry1Ac transgenic lines to verify gene insertion, determine the number of inserted copies of the gene, and quantify the amount of protein; and 3) assess the potato tuberworm response on Bt cry1Ac transgenic lines.
Materials and Methods

Vector construct.

The vector pSP 73 cry1Ac improved, kindly supplied by Dr. John Kemp, New Mexico State University, was digested with XhoI and then treated with T4 DNA polymerase to create blunt ends. It was 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 EsclCR1. The resulting plasmid is pSPUD15 (Fig. 1).

Plant material.

Two potato lines were used for the Agrobacterium-mediated transformation experiment: Spunta and ND5873-15. Spunta is a long, light-yellow fleshed, tablestock cultivar that was bred in the Netherlands. It is grown widely in subtropical regions, such as North Africa and South America (Douches et al., 2002). ND5873-15 is a breeding line from North Dakota State University (NDSU) that has higher levels of an uncharacterized resistance factor in the foliage derived from the wild relative S. chacoense (Dr. J. Lorenzen, pers. comm.). Transgenic lines derived from Spunta and ND5873-15 were named sequentially Sp15.X and ND15.X, respectively.

Transformation protocol.

Agrobacterium tumefaciens-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 generation of the transgenic lines of Spunta and ND5873-15. Potato lines were prepared for transformation by removing the tip and the petiole ends from tissue culture plantlet leaves. All tissue culture and transgenic lines grown were analyzed by Southern analysis. Integration and copy number of the Bt 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, Valencia, CA). The primers used for the Bt cry1Ac gene were 5′ CATGCTCTGAGA CCGGTGTAACCTC 3′ and 5′ CTGTCTA TGATCACACCTGACGTTC 3′. The expected band size was 1.8 kb. 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 s, and primer extension at 72 °C for 1 min 30 s, and a final extension at 72 °C for 4 min. The reactions were held at 4 °C before the analysis. Reaction products were electrophoresed on a 1% (w/v) agarose gel containing ethidium bromide at 0.5 mg mL⁻¹ in 1× Tris-acetate EDTA buffer (pH 8.0) at 80 mV for 1 h 30 min and viewed under ultraviolet light (254 nm).

Southern analysis. Integration and copy number of the Bt cry1Ac gene in the transgenic plants were analyzed by Southern analysis. Total DNA was isolated from the leaf tissue using the CTAB extraction protocol (Saghi-Maroof et al., 1984), modified by adding 2% β-mercaptoethanol to the extraction buffer. DNA was digested using XhoI (Roche, Roche, Penzberg, Germany). The digested DNA fragments were then electrophoresed in a 1.2% (w/v) agarose gel. 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 was conducted overnight at 42 °C in a fresh solution containing =25 ng mL⁻¹ DIG-labeled DNA probe. CSPD chemiluminescent detection was conducted following the manufacturer’s procedures using 75 mM L⁻¹ antidigoxigenin alkaline-phosphatase conjugate and 1 mL of CSPD ready-to-use substrate (Roche, Mannheim, Germany). The membrane was then exposed to X-ray film (Hyperlens MP, Amersham Life Sciences) for 15–30 min and developed.

ELISA assay. The Bt cry1Ac protein expressed in each transgenic plant was detected using the double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) for Bt-Cry1Ab/1Ac proteins kit from Agdia (Elkhart, IN). Leaf samples were taken from greenhouse-grown plants and were homogenized using Agdia mesh-lined tissue sample bags. The ELISA protocol was done according to the manufacturer’s instructions. The optical density was measured using the Wallac Victor II plate reader (PerkinElmer Life Sciences, Downers Grove, IL) at 450 nm. A standard curve was constructed from the controls and was used to compute the Bt Cry1Ac protein expression in ng/mL.

Potato tuberworm rearing.

A potato tuberworm population from South Africa has been maintained at the Department of Entomology, Michigan State University, since 2004 on potato tubers following the rearing method described by Mohammed et al. (2000).

Detached-leaf bioassay.

Fully expanded leaves were collected from greenhouse-grown plants of each potato line. Each petiole was immersed in a water filled vial (4 mL) sealed with parafilm and placed into a petri dish (125 mm diam.) lined with Whatman No. 2 filter paper (Douches et al., 1998). Ten newly hatched first-instar larvae were placed on each detached-leaf for 3 d in a no-choice test. The assays were conducted at room temperature (22 ± 2 °C) under fluorescent lights on a 16 h/8 h light/dark schedule. The assays were replicated four times, with each potato line represented in each replicate to compensate for any variance in plant quality between replicates. After 3 d, the mortality was measured. Larvae were extracted from the leaf mine under a dissecting microscope and considered dead if no movement was observed after being lightly touched with a small paintbrush. For the high-expressing Bt cry1Ac lines, ND15.11 and ND15.10, we repeated the experiment with ND5873-15 as a control but extended the duration of the assay to 5 d to better characterize the resistance reaction for mortality.

Tuber bioassay.

Tubers were harvested from greenhouse-grown plants. A single tuber was placed inside a closed container with vermiculite and

Fig. 1. Schematic diagram of the pSPUD15 vector construct. Abbreviations: RB, right border of T-DNA; NOS-Ter, NOS terminator; cry1Ac, Bt cry1Ac insecticidal protein gene; CaMV 35S Pro, promoter; nptII, neomycin phosphotransferase gene confers resistance to kanamycin; NOS-Pro, NOS promoter; LB, left border of T-DNA.
was exposed to 10 first-instar larvae. Neonates were placed on the tuber eyes with a paintbrush. Each container was considered as a replication. Four replications per transgenic line were used, and the containers were arranged in a completely randomized design in an insect-rearing room (25 ± 2 °C and 70 ± 5% relative humidity). After 21 d, the tubers were cut into sections, the numbers of pupae were recorded, and the overall mortality was expressed as percentage.

Statistical analysis.
Percentage mortality data for both leaf and tuber bioassays were subjected to arcsine transformation before testing one-way analysis of variance (ANOVA) (SAS Inst., 2001). Means were compared using Fisher’s least significant difference (LSD) in the general linear model procedure (α = 0.05) of SAS (SAS Inst., Inc., 2001). All results are presented as percent, converted back from the arcsine transformation.

Results

Potato transformation with \( Bt \) cry1Ac gene.
A total of 32 putative transgenic cry1Ac potato shoots were produced from Spunta leaf explants. Of these 32 shoots, 12 rooted in the kanamycin (50 mg/L) media. These 12 shoots were then regenerared into plants. Eleven of the 12 rooted plants were positive for the presence of the \( Bt \) cry1Ac gene via PCR.

ND5873-15 required 8–10 weeks to produce shoots compared with 5–8 weeks for Spunta. A total of 34 putative transgenic potato shoots were regenerated. Of these 34 shoots, 12 rooted in the Kanamycin media from ND5873-15. Eight of the 12 rooted shoots were confirmed PCR positive for the presence of the \( Bt \) cry1Ac gene.

Molecular characterization.

PCR analysis. All the lines that tested PCR positive were subjected to ELISA for protein expression and detached-leaf bioassays. High-performing lines from ND5873-15 and from Spunta were identified from the detached-leaf bioassay (below). These were further characterized using Southern analysis.

Southern analysis. Southern analysis revealed that there was one copy of \( Bt \) cry1Ac inserted in all the lines except for ND15.10 and Sp15.07, which had two copies (Fig. 2).

ELISA assay. \( Bt \) Cry1Ac protein expression in Spunta and ND5873-15 transgenic lines varied between 0 to 580 ng/g of fresh leaf tissue (Table 1). In general, the level of protein expression in the ND5873-15 \( Bt \) cry1Ac lines was higher than the level in the Spunta \( Bt \) cry1Ac lines. The controls, Spunta and ND5873-15, tested negative for the \( Bt \) Cry1Ac protein expression.

Detached leaf bioassay.
The analysis of variance (ANOVA) for the overall model was significant for each of the detached leaf tests and the tuber bioassays. The mortality of larvae feeding on non-transgenic Spunta lines (3%) was significantly lower than the mortality of larvae feeding on any transgenic Spunta line expressing \( Bt \) cry1Ac (22–58%; \( P < 0.01 \); Fig. 3). There was a significant correlation between protein expression and mortality of larvae feeding on transgenic Spunta expressing \( Bt \) cry1Ac (\( r^2 = 0.40, P = 0.02 \)).

The mortality of larvae feeding on non-transgenic ND5873-15 (35%) did not differ significantly compared with the mortality of larvae fed on transgenic ND5873-15 lines with lower \( Bt \) Cry1Ac expression (<100 ng/g) of fresh tissue (ND15.01, ND15.05, ND15.07, ND15.08, ND15.12, and ND15.13) (\( P < 0.01 \)) but was significantly higher than Spunta (Fig. 3). In contrast, the mortality of larvae feeding on non-transgenic ND5873-15 was significantly lower than mortality of larvae fed on transgenic ND5873-15 with higher expression (>100 ng/g) of fresh tissue (ND15.07, ND15.10, and ND15.11). Hence, there was a significant correlation between protein expression and mortality of larvae fed on transgenic ND5873-15 expressing \( Bt \) Cry1Ac (\( r^2 = 0.50, P = 0.03 \)).

Comparing ND5873-15 and Spunta \( Bt \) cry1Ac transgenic lines with similar expression levels (<100 ng/g), the mortality of larvae fed on non-transgenic ND5873-15 lines did not significantly differ from the mortality of larvae fed on transgenic Spunta lines (\( P < 0.01 \)) (Fig. 3).

In the 5-d detached leaf bioassay evaluation, the mortality of larvae fed on non-transgenic ND5873-15 (17%) was significantly lower than mortality of larvae fed on transgenic ND15.10 (88%) and ND15.11 (97%) (\( P < 0.01 \)) (Fig. 4).

Tuber bioassay.
Two \( Bt \) cry1Ac-expressing lines (ND15.10 and ND15.11) were selected for the tuber bioassay due to the high levels of control in the leaf bioassay. The mortality of larvae fed on non-transgenic ND5873-15 (22%) was significantly lower than mortality of larvae fed on transgenic ND15.10 (97%) and ND15.11 (99%) (\( P < 0.01 \)) (Fig. 4).

Discussion

Potato transformation with \( Bt \) cry1Ac 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 by expressing \( nptII \) was effective for Spunta and ND5873-15 and made the number of test samples manageable. Fewer transgenic lines were produced with ND5873-15 than with Spunta, and ND5873-15 lines took longer to produce shoots. Differences in the ease of producing transgenic lines between cultivars were also observed by Felcher et al. (2003).

![Southern analysis of ND5873-15 and Spunta Bt cry1Ac lines to determine the number of gene copies inserted.](image-url)
In their study, they recovered a significantly higher number of transformed Spunta and Atlantic glucose oxidase transgenic lines compared with 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 fewer rooted shoots in the kanamycin (50 mg/L) medium compared with the other two lines. These differences in the successful regeneration of transgenic shoots between cultivars/lines suggest that there is genetic variation for regeneration and transformation efficiency.

Southern analysis showed that 1–2 copies of the Bt cry1Ac gene were inserted in the Spunta and ND5873-15 lines. 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 the Bt cry1Ac9 gene. Douches et al. (1998) reported one to three copies of Bt cry1Al. 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 Bt cry1Ac9 gene inserted in tobacco transgenic lines.

The high mortality of potato tuberworm larvae fed on ND15.11 and ND15.10 corresponded to their high Bt Cry1Ac protein expression. The ability to achieve high protein expression from both lines may be due to the synthetic codon-modified cry gene used which can give expression that is significantly higher compared with expression of the 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 expression from cry gene vector constructs are about 0.1% to 0.3% of soluble protein corresponding to about 1 μg of toxin protein per g of fresh leaf tissue (Perlak et al., 1993; Kuvshinov et al., 2001). Variation in the level of resistance to potato tuberworm was observed from the independently derived Spunta and ND5873-15 Bt cry1Ac transgenic lines. Such variation in the level of transgene expression is common among populations of plants that are independently transformed with the same transgenes. In this study, similar mortality was observed for many of the Spunta and ND5873-15 transgenic lines. This could be primarily attributed to the Cry1Ac protein levels. This unpredictable expression may have led to greater mortality than was observed by Davidson et al. (2002). Kuvshinov et al. (2001) observed a significant correlation between mortality in the feeding assay and for protein expression for both ND5873-15 and Spunta Bt cry1Ac 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 stopped feeding as soon as they became intoxicated. Larvae feeding on potato foliage with low concentrations of Bt toxin may have entered into prey as they became intoxicated. Larvae feeding on potato foliage with low concentrations of Bt toxin may have fed longer and consumed more foliage compared with the amount consumed by larvae feeding on foliage with high concentrations of Bt toxin.

The resistance provided by the integration of Bt cry1Ac in this study was enhanced in both the Spunta and ND5873-15 transgenic lines (Fig. 3); however, the resistance level observed for the best Spunta lines was not different from the best ND5873-15 transgenic lines. In this study, potato tuberworm mortality was similar to the resistance provided.
by the integration of Bt cry1Aa in the study conducted by Westedt et al. (1998). They observed that potato transformation with a codon-modified Bt cry1Aa provided up to 96% mortality of potato tuberworm. In our study, potato tuberworm mortality was up to 97% (Fig. 4). In their study, Mohammed et al. (2000) observed higher potato tuberworm mortality, up to 100% in the Bt cry1Aa Spunta lines, and only partial resistance using a wild-type Bt cry1Ac gene. Similarly, 100% potato tuberworm mortality after 10 d was observed with Bt cry1Ac9 potato lines (Davidson et al., 2002). Janssen et al. (1995) observed protein content of 3–11 ng toxin/mg total protein in the leaves in the Yesmina and Kennebec Bt cry1Ac lines and 40–100% potato tuberworm mortality. Davidson et al. (2004) achieved only reduced growth of larvae on potato foliage expressing Bt cry1Ac9.

The low mortality in NDS873-15 (22%) in spite of the fact that it has natural resistance may be attributed to the level of glycoalkaloid content in the leaves used in the detached leaf bioassays (Fig. 3). The glycoalkaloid concentration in potato plants can be highly affected by the temperature, light intensity, and daylength. Glycoalkaloids are present at higher concentration in the aerial (leaves, stems, and sprouts) parts of the potato plant and are normally present in lower concentration in the tubers (Lachman et al., 2001). Similarly, Lafla and Lorenzen (2002) observed a significant increase in foliar glycoalkaloids when plants were grown at higher temperatures (32 vs. 27 °C). The higher mortality observed in NDS873-15, compared with the Spunta control, is probably the result of the uncharacterized natural insect resistance factors in NDS873-15 (Dr. J. Lorenzen, pers. comm.).

Higher mortality observed in the 5-d detached leaf bioassay with ND15.10 and ND15.11 compared with the mortality on the detached leaves healthy for that period may set practical limits to this assessment. The production of more independently selected Bt cry1Aa lines will allow recovery of lines with high transgene expression, as well as phenotypically normal appearance and yield performance (Meiyalaghan et al., 2004). 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 Bt cry1Aa in their progeny is also of value. The Bt cry1Ac gene can be another source of resistance that can be combined with other genes (natural or engineered) for host plant resistance to potato tuberworm in potato.

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