Evaluation of Natural and Engineered Resistance Mechanisms in *Solanum tuberosum* for Resistance to *Phthorimaea operculella* (Lepidoptera: Gelechiidae)

A. L. WESTEDT, D. S. DOUCHES,[1] W. PETT, AND E. J. GRAFIUS

Department of Crop and Soil Sciences and Department of Entomology, Michigan State University, East Lansing, MI 48824

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ABSTRACT Potato tuber moth, *Phthorimaea operculella* Zeller, is a highly destructive pest of cultivated potato, *Solanum tuberosum* L., and is responsible for damage to both leaf and tuber tissues. Host plant resistance is a central component to developing an integrated pest management program to control potato tuber moth. This research tested the efficacy of a codon-modified CryV-*Bacillus thuringiensis* (CryV-Bt) gene constitutively expressed in potato and the combined effect of CryV-Bt expression with natural host plant resistance mechanisms in potato. 'Lemhi Russet' and 2 lines with host plant resistance mechanisms, USDA8380-1 (leaf leptines) and L235-4 (glandular trichomes), along with the CryV-Bt-transgenic lines of each of these 3 genotypes were examined. Detached leaf bioassays were conducted to examine control of potato tuber moth. Nontransformed Lemhi Russet and L235-4 were susceptible to potato tuber moth, while 54% potato tuber moth mortality was found when first instar larvae fed on USDA8380-1 leaves. High levels of expression occurred in the CryV-Bt transgenic lines, with up to 96% potato tuber moth mortality. These transgenic lines provide a germplasm base to examine combined insect-resistance mechanisms as a means to achieve durable host plant resistance.

KEY WORDS *Bacillus thuringiensis*, genetic engineering, leptine, glycoalkaloid, glandular trichome

INSECTS, VIRUSES, DISEASES, and abiotic stresses are some of the many constraints to potato production. One of the most important insect pests in the cultivated potato, *Solanum tuberosum* L., worldwide is the potato tuber moth, *Phthorimaea operculella* Zeller, which causes damage in both field and storage. Geographic regions with average daily temperatures >16°C experience the greatest damage by the potato tuber moth (Raman and Palacios 1982).

Potato tuber moth larvae attack the potato by mining in the leaves or the tubers (Raman 1980, Goldson and Emberson 1985, Trivedi and Rajagopal 1992). Larvae penetrate and feed on the leaves in addition to tunneling within leaf veins and stems of the plant. This damage causes loss of leaf tissue, death of growing points, and weakening or breakage of stems (Bald and Helson 1944, Raman 1980). The tubers are infested by larvae emerging from eggs deposited on the surface of the soil near the stem, in cracks in the soil, or laid near the tuber eyes when in storage. The larvae mine into the tuber causing irregular tunnels both near the surface and deep inside the tuber, rendering them unfit for human consumption. When potatoes are not available, potato tuber moth larvae may feed on other Solanaceae including tobacco, *Nicotiana tabacum* L., tomato, *Lycopersicon esculentum* Mill., and eggplant, *Solanum melongena* variety (Goldson and Emberson 1985). Although the potato tuber moth is not strictly confined to tuber feeding, it is unable to rapidly multiply if limited to leaf mining alone (Trivedi and Rajagopal 1992).

Host plant resistance is a desired central component of an integrated pest management (IPM) program to control the tuber moth. Two major forms of host plant resistance, antixenosis and antibiosis, have been described for potato. Morphological antixenosis is resistance due to a structural feature that impairs the normal processes of insects (Kogan 1982). An example would be high densities of glandular trichomes such as those found in the wild potato species *S. berthaultii* Hawkes, *S. polyadenium* Greenman, and *S. tarijense* Hawkes (Tingey et al.1984). The defensive system of *S. berthaultii* results in small-bodied insects exhibiting modified behavior, including host avoidance and restlessness, reduced feeding, delayed development, and diminished longevity (Tingey 1991). Reduced pupation by tuber moth has been attributed to antibiosis (resistance due to chemical factors) in potato tubers of primitive cultivars (Raman and Palacios 1982). Chavez et al. (1988) reported that this type of resistance to potato tuber moth was transferred to all progeny except hybrids where *S. tarijense* was the resistant parent.

[1] Current address: David Douches, Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI 48824-1325. douchesd@pilot.MSU.edu

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Glycoalkaloids are the most common form of antibiosis in potato (Sinden et al. 1986). Steroidal glycoalkaloids (solanine and chaconine) are present in potato tubers and processed products (Sinden 1987). Glycoalkaloids below 20 mg/100 g (milligram percentage) fresh weight in the tuber are considered safe for human consumption (Sinden and Webb 1972). *S. tuberosum* generally contains only 2–10 mg percentage of glycoalkaloid; however, these levels are greatly influenced by genetic and environmental conditions. Factors such as soil type, soil moisture, fertilizer level, pesticides, light quality and quantity, and mechanical damage all may contribute to increased levels of glycoalkaloids in the tuber. Wild potato species such as *S. chacoense* Bitter and *S. commersonii* Bitter can have concentrations of 230 mg percentage and 500 mg percentage of glycoalkaloids, respectively (Sinden 1987).

Bacillus thuringiensis (Bt) toxin genes have been cloned, codon-modified, and inserted in various crop species (Barton and Miller 1993). Since the initial cloning work with Lepidopteran-specific Bt, other Bt genes have been isolated and cloned that have specificities for Coleoptera (beetles) and Diptera (flies). Transformations of potato with a wild-type Cry IA Bt toxin gene specific for Lepidoptera have produced low levels of Bt expression with 20–60% insect mortality in detached leaf bioassays (P. Hudy and D.S.D., unpublished data). These levels of expression for wild type genes are consistent with previous results (Barton et al. 1987, Ebora et al. 1994, Wünn et al. 1996). The CryV Bt gene, effective against both Lepidoptera and Coleoptera, has been codon-modified to increase expression in plants. The efficacy of other codon-modified Bt genes such as Cry I and Cry III Bt is greater than the efficacy of wild type Bt in crop plants (Perlak et al. 1990, Wünn et al. 1996). Unfortunately, there is evidence that insects can adapt to Bt toxins, reducing or eliminating their effectiveness (Whalon et al. 1993).

Douches et al. (1998) combined natural resistance mechanisms with the CryV-Bt gene, by *Agrobacterium*-mediated transformation, to confer host plant resistance to the potato tuber moth. The objective of this research is to determine if pyramiding resistance mechanisms leads to a more durable host plant resistance. We report on the evaluation of (1) 'Lemhi Russet' (susceptible) and 2 potato genotypes with natural resistance mechanisms (glandular trichomes or foliar leptines) and (2) CryV-Bt transgenic potato lines derived from the 3 potato genotypes above to confer resistance to potato tuber moth in potato using detached leaf bioassays.

Materials and Methods

Vector Construct. The codon-modified Cry V-Bt gene (2,200 bp) was obtained from Zeneca/ICI Seeds (Berkshire, UK) and was ligated into the BamHI site of pBI121 (Clontech, Palo Alto, CA) (Douches et al. 1998) (Fig. 1). This vector construct (pBICryV) was transformed into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating (Ditta et al. 1980).

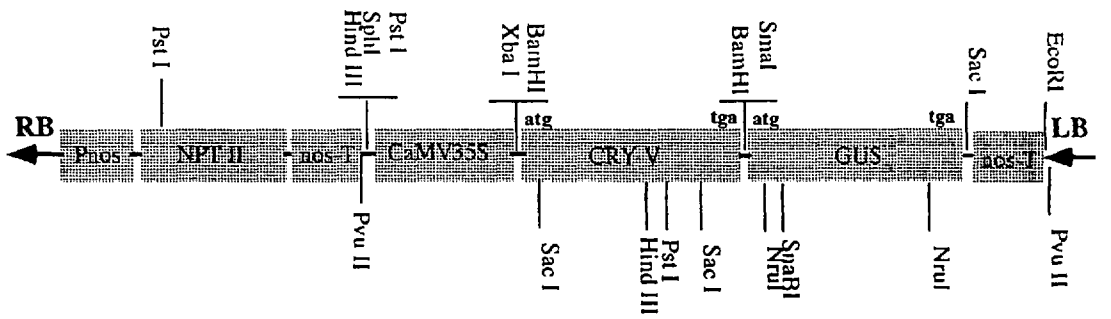
Plant Materials. The potato lines used for transformation experiments and potato tuber moth bioassays were USDA8380-1, a *S. chacoense* selection obtained from USDA–ARS Beltsville; L235-4, from Cornell University, and Lemhi Russet. The first 2 genotypes were chosen because of their natural host plant resistance factors: USDA8380-1 (high concentration of leaf leptines) (Sinden et al. 1986) and L235-4 (glandular trichomes) (Plaisted et al. 1992). Lemhi Russet is a potato tuber moth susceptible commercial variety with high regeneration ability in tissue culture.

Potato Tuber Moth Rearing. *Egg Production.* Potato tuber moth cultures were obtained from Lowell Etzel, University of California, Berkeley. Tuber moth pupae were placed into a 3.785-liter glass jar with a 50% honey/water solution as a food source in a 36-ml souffle cup, which was present throughout the egg laying period. The glass jar was covered with nylon window screen to provide a landing surface for oviposition. A No.1 Whatman filter paper (150 mm diameter) was placed over the screen to serve as a surface for egg laying. The cultures were maintained at 25°C in complete darkness.

Larva Through Pupa Development. Filter papers with eggs were collected every 2 d and placed on potato tubers or used for plant assays. The tubers were kept in a plastic tub (45 by 30 by 20 cm^3) at 26°C in complete darkness. The tubs were covered with a double layer of cheesecloth to prevent escape of larvae but still allow air exchange. Newly hatched larvae were allowed to mine the tubers for a food source. Corrugated cardboard was cut into 5-cm squares, stacked 5 high and placed in the tubs to allow a refuge for pupation. Fully developed larvae would leave the tubers and enter the pupal chambers for pupation. Pupae were collected and moved to clean 3.785-liter glass jars to continue the process.

Potato Tuber Moth Bioassay. All transgenic and non-transgenic potato lines were maintained in tissue culture and used for production of greenhouse plants for bioassays. A detached leaf bioassay was used to test for feeding efficacy of the Cry V-Bt transgenic plants. Leaf tissue was collected in the morning by cutting off a young, fully-expanded leaf from 4- to 8-wk-old greenhouse plants. The petiole of the leaf was cut off under water using a new single-edged razor blade. The petiole was inserted in a slit in a premoistened half sponge (1 cm diameter, 1 cm long) and then placed in a 3.5 ml glass vial full of water. The leaf and the vial were then placed on a premoistened filter paper disk (150 mm diameter) enclosed inside a disposable petri dish (150 by 20 mm) and labeled with the genotype.

Potato lines (18 Lemhi Russet CryV-Bt, 25 USDA8380-1 CryV-BT, and 11 L235-4 CryV-Bt lines), including a susceptible control, were tested in preliminary bioassays (2 trials, 5 first-instar larvae per leaf). The larvae were placed on the leaves near the mid-rib. The disposable petri dishes with the enclosed leaves were set on a 25 8μE m^{-2} s^{-1} lighted lab bench kept at 25°C ± 2. After 48 h, survival/mortality of the potato tuber moth larvae were recorded. Lines with >80% mortality in these preliminary feeding trials were selected for final testing (10 first-instar larvae per



name: **pBICry V**
source info: Cry V gene (in pBS) inserted into pBI121
length info: **Pnos** 306bp
NPT II 794bp
nos-T 252bp
CaMV35S 872bp
Cry V 2200bp
GUS 1811bp
nos-T 320bp
Frame length 7883bp

Fig. 1. Characteristics of the codon-modified Cry V Bt gene.

leaf; 3–8 replications). Significance was determined with a Wilcoxon test (SAS 1989).

Results

A total of 18 polymerase chain reaction (PCR)-positive Lemhi Russet CryV-Bt lines and the nontransgenic Lemhi Russet were used in preliminary potato tuber moth bioassays. Mortality ranged from 20 to 95% mortality among the transgenic lines versus 5% on the nontransgenic Lemhi Russet. Eight lines that demonstrated a mortality of ≥80% were used for final testing. Mortality on foliage from the transgenic lines was 83–93% versus 3% nontransformed Lemhi Russet (Table 1).

Twenty-five CryV-Bt transgenic lines of USDA8380-1 were also tested in a preliminary feeding trial. The nontransgenic USDA8380-1 had 50% potato tuber moth mortality in the initial bioassay versus 40–100% on the USDA8380-1 Cry V-Bt lines. The 7 USDA8380-1 CryV-Bt lines with the highest mortalities were tested further. All these transgenic lines had significantly higher potato tuber moth mortality in the final trial than USDA8380-1 (Table 1).

Untransformed L235-4 and 11 L235-4 CryV-Bt lines were used in preliminary potato tuber moth feeding bioassays. The best 8 lines were selected from these tests for further potato tuber moth bioassays. Potato tuber moth mortality in final trials ranged from 88 to 96% in the L235-4 CryV-Bt lines; all L235-4 CryV-Bt lines had higher potato tuber moth mortality than on the untransformed L235-4 (Table 1).

Discussion

The overall purpose of this study was to combine natural host-plant resistance mechanisms with the CryV-Bt gene by transformation, pyramiding host plant resistance mechanisms against potato tuber moth. In detached leaf tests, we observed a strong effect of the CryV-Bt gene to control potato tuber moth with up to 90% greater potato tuber moth mortality. Foliar leptines in USDA8380-1 caused 54% mortality, whereas no effect of glandular trichomes was observed in these detached leaf tests.

Examining Bt efficacy in the USDA8380-1 CryV-Bt lines was problematic. The leaf bioassay could not distinguish between Bt and leptine effects. After 72 h, the leaves of the high-leptine line USDA8380-1 caused 70–100% mortality of potato tuber moth (W.P., unpublished data). These levels of mortality were also typical of the insect mortalities observed with Lemhi Russet CryV-Bt lines. The potato tuber moth bioassays were shortened to 48 h to reduce the effect of the leptines on the potato tuber moth mortality relative to

Table 1. Mortality (48 h) of PTM larvae on foliage in detached leaf bioassays

Line	Mortality, %[a]	SD
Lemhi Russet Cry V-Bt transgenic lines[b]		
Lemhi Russet	3	4.7
Lemhi-1	90	2.7
Lemhi-7	93	2.7
Lemhi-12	83	2.7
Lemhi-14	93	2.7
Lemhi-15	83	2.7
Lemhi-17	88	3.9
Lemhi-21	86	2.7
Lemhi-22	88	3.9
USDA8380-1 Cry V-Bt lines[c]		
USDA 8380-1	54	9.1
8380-1.1	75[d]	9.3
8380-1.5	78[d]	9.3
8380-1.9	88[d]	9.3
8380-1.16	80[d]	9.4
8380-1.18	78[d]	8.5
8380-1.19	69[d]	9.2
8380-1.25	87[d]	9.3
8380-1.26	78[d]	9.3
L235-4 Cry V-Bt transgenic lines[e]		
L235-4	4	8.0
L235-4.3	96	4.6
L235-4.5	90	4.6
L235-4.8	96	4.5
L235-4.11	90	4.6
L235-4.12	90	4.6
L235-4.13	96	4.5
L235-4.14	88	4.6
L235-4.16	96	4.5

[a] Significantly different from untransformed line, $P < 0.01$, Wilcoxon test.
[b] Five replications per treatment.
[c] Eight replications per treatment.
[d] Significantly different from untransformed line, $P < 0.05$, Wilcoxon test.
[e] Three replications per treatment.

control due to CryV-Bt. The reduction in time reduced the mean mortality in USDA8380-1 due to leptines alone to 54%. This mortality level was low enough to allow distinction between CryV-Bt lines. When a few detached leaf assays were continued for 72 h, 100% insect mortality was usually observed (unpublished data).

Since somaclonal variation can occur from the transformation or regeneration process, we cannot assume that the leptine levels in the USDA8380-1 CryV-Bt lines are similar. Further investigation into CryV-Bt expression and leptine production in the transgenic lines would be useful. Biochemical characterization should be done to determine actual Bt and leptine levels. Western blot analysis and high-pressure liquid chromatography (HPLC) could be used to estimate CryV-Bt protein levels and leptine levels (Sinden 1986), respectively, in these transgenic lines.

The CryV-Bt differs from the other Bt constructs in that it is specific for Coleoptera and Lepidoptera insects. The potato tuber moth mortalities using our CryV-Bt lines were as high or higher than those observed for a codon-modified CryIA Bt gene (Wünn et al. 1996). CryIA is also active against other Lepidoptera. An additional value of the CryV-Bt construct is that is potentially active against Colorado potato beetle, *Leptinotarsa decemlineata* (Say). However, control of Colorado potato beetle is an order of magnitude lower with the CryV-Bt than with the CryIII-Bt (T. Kisha, D.S.D., and W.P., unpublished data).

Our construct had the β-glucuronidase (GUS) gene fused to the CryV-Bt gene, with a single 35S promoter controlling both genes. It has been observed that the GUS gene may interfere with gene expression in potato (Belknap et al. 1994). This research demonstrates that high levels of expression of the CryV-Bt can be obtained, despite northern blot analysis showing that the GUS gene was being transcribed with the Bt gene (Douches et al. 1998). Our next step is to compare the expression of CryV-Bt constructs in potato with and without the GUS gene.

The results from the CryV-Bt transgenic lines of Lemhi Russet and L235-4 demonstrated that high levels of CryV-Bt expression can be achieved with this gene construct. If Bt expression is to be used as a tool in host plant resistance management strategies, the next step is to look at how the lines can be used in a potato tuber moth resistance management strategy. Host plant recognition by phytophagous insects, such as potato tuber moth, occurs in 5 stages: (1) host-habitat finding, (2) host finding, (3) host recognition, (4) host acceptance, and (5) host suitability (McGaughey and Whalon 1992). If the process is disrupted, then the host is not accepted (Kogan 1982). Both the trichomes and leptines affect the potato tuber moth at the host acceptance stage (Sinden et al. 1986, Yencho and Tingey 1994). Under field conditions, these natural host plant mechanisms will reduce the number of potato tuber moth accepting the plant as a host and ovipositing, reducing the number of insects being selected for adaptation to CryV-Bt. Reduction in Bt selection pressure on the potato tuber moth will reduce selection intensity for resistance genes in the potato tuber moth population. If some potato tuber moth larvae die due to the type A glandular trichomes, then the number of potato tuber moth on a potato plant is further reduced, slowing the insect adaptation to Bt. Some USDA8380-1 CryV-Bt lines showed lower potato tuber moth mortality levels than untransformed. This may be caused by a reduction of leptine production which may interfere with the deterrent nature of the host plant or the effectiveness of the CryV-Bt gene. It is possible that the low levels of Bt toxin production may have reduced potato tuber moth feeding which would reduce leptine ingestion, allowing survival.

The leaf bioassay utilized for these studies and high levels of Bt expression in the leaf were demonstrated; however, the tuber is the primary economically damaged tissue. The next key question to be asked is whether the potato tuber moth is controlled by CryV-Bt in the tuber; further investigation will include tuber bioassay and field studies. The constitutive expression promoter, Cauliflower Mosaic Virus 35S promoter (CaMV 35S) was used to obtain CryV-Bt expression (Benfey and Chua 1990). Bioassays to determine if Bt gene expression in the tuber correlates with expression in the leaf will be of value. Some

preliminary investigations into tuber and leaf mortality correlation studies have been attempted, but further research is needed (data not shown). Future work will include testing of the CryV-Bt gene in combination with the Gelvin super promoter (Narasimhulu et al. 1996) and the patatin promoter (Wenzler et al. 1989) for expression levels. In addition, field studies are needed to compare the value of combined engineered and natural host plant resistances to potato tuber moth for the various genotypes.

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