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Genetically improved potatoes: protection from damage by Colorado potato beetles

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Abstract

Russet Burbank potato plants have been genetically improved to resist insect attack and damage by Colorado potato beetles (*Leptinotarsa decemlineata* (Say)) by the insertion of a *cryIIIA* gene encoding the insect control protein of *Bacillus thuringiensis* var. *tenebrionis*. A modified gene that dramatically improved plant expression of this protein was utilized. Its expression in Russet Burbank potato plants resulted in protection from damage by all insect stages in the laboratory and in dramatic levels of protection at multiple field locations. Analysis of these genetically modified potatoes indicated that they conform to the standards for Russet Burbank potatoes in terms of agronomic and quality characteristics including taste.

Introduction

Colorado potato beetles (CPB; *Leptinotarsa decemlineata*) are the most destructive pests of potato. The life cycle of this insect, its destructive feeding habits and its demonstrated ability to develop resistance to synthetic chemical insecticides have made control of CPB an increasing agricultural problem [9]. The United States Department of Agriculture estimates that potato growers spend $75 to $100 million annually to control CPB on ca. 480 000 ha of potatoes. *Bacillus thuringiensis* var. *tenebrionis* (*B.t.t.*) produces a parasporal crystal protein, CryIIIA, that is insecti-

The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X70979.

cidal to CPB [17]. This protein is characterized by its high unit activity and specificity for certain coleopteran insects including CPB. CryIIIA has been reported to form cation-selective channels in lipid bi-layers similar to the ion selective channels formed by CryIA(c) [29]. We and others have previously cloned, sequenced and characterized the *cryIIIA* gene that encodes the CryIIIA insecticidal protein [12, 14, 21, 27]. The *cryIIIA* genes described by us and these other groups are identical in sequence. Expression of this gene in transgenic plants could provide a novel and powerful method for CPB control. However, expression of other insecticidal protein genes from *B. thuringiensis* (*B.t.*) has been problematic in plants [2, 8, 31]. We previously reported that modification of the coding sequence dramatically increases the expression (up to 500-fold compared to the wild type gene) of the lepidopteran specific *B.t.* genes *cryIA(b)* and *cryIA(c)* in plants [24, 25]. These modified forms of the lepidopteran-active *cryIA* genes have been expressed in cotton, and these cotton plants show a high level of protection from insect damage in the laboratory and the field [32]. Others have now reported improved plant expression through modification of *cryIA* genes, but detailed sequence information on these genes has not yet been published [1, 6, 19]. We also described a general methodology for modifying *B.t.* genes to improve plant expression. Using that methodology, initially developed for *cryIA* genes, we have now modified the coleopteran-active *cryIIIA* gene. We report here that this modified form of the *cryIIIA* gene is expressed at high levels in transgenic potato plants, and that this expression confers a very high level of protection from damage by CPB in both the laboratory and the field. Recently, Sutton *et al.* [30] reported that a modified *cryIIIA* gene was expressed efficiently in tobacco plants.

Results and discussion

Preliminary experiments in which the wild-type form of the *cryIIIA* gene was introduced into tomato and potato plants indicated that the wild-type *cryIIIA* gene was also poorly expressed in plants [26]. Plants containing this gene expressed CryIIIA protein at less than 0.001% of total leaf protein, although *cryIIIA* mRNA was detectable by northern analysis. This expression level from the wild-type *cryIIIA* is similar to expression levels from truncated forms of the wild-type *cryIA* genes. While these plants exhibited toxicity to Colorado potato beetle larvae, our previous experience with *cryIA* genes in tomato and cotton [5, 24] indicated that higher levels of expression might be required for consistent protection from insect damage in the field. To achieve this, a modified version of this *cryIIIA* gene was constructed by extensively modifying the DNA protein-coding sequence without altering the amino acid sequence.

This coding sequence modification strategy was initially developed for and applied to the lepidopteran-specific *cryIA(b)* and *cryIA(c)* genes, which share only about 25% DNA sequence homology to the *cryIIIA* gene. The *cryIIIA* shares several common sequence motifs with the *cryIA* genes that were the targets of modification. These motifs include a high A + T content, ATTTA sequences identified as responsible for the destabilization of mRNA in animal cells [28], potential plant polyadenylation sequences [4] and long stretches composed solely of A + T which may resemble plant introns [11]. All of these sequence motifs were targets for modification in *cryIA* genes as previously described by us. In that report [24] we showed that selective modification of these motifs, in the partially modified *cryIA(b)* gene, gave a dramatic increase in plant expression, even when fewer than 10% of total codons were changed. Fully modified versions of *cryIA(b)* and *cryIA(c)* genes provided even higher levels of expression, so the modified *cryIIIA* gene of this paper was modeled on those fully modified genes. Figure 1 shows the DNA sequence of the *cryIIIA* gene improved for plant expression and constructed by total gene synthesis, which was introduced into transgenic plants. This gene begins at amino acid 48 of the full-length gene, an internal methionine codon used as a second translational initiation site in *E. coli* and probably in *B.t.t.* as

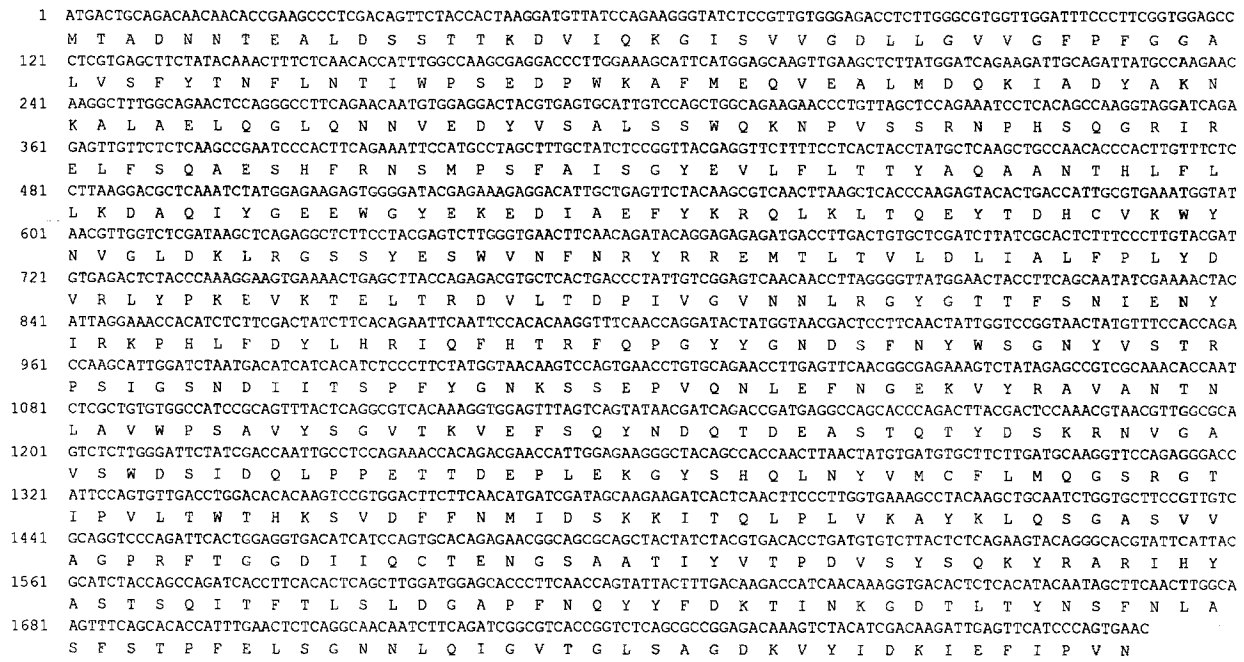

Fig. 1. The DNA sequence of the modified *cryIIIA* insect control protein gene and the protein encoded. The amino acid sequence is identical to the amino acid sequence of the protein produced in *Bacillus thuringiensis* var. *tenebrionis*.

well [21, 27]. This protein has been shown to be as effective in insect control of CPB as the full-length protein. Table 1 summarizes several of the important differences between the wild-type and modified form of *cryIIIA*, focusing particularly on those sequence motifs described by Perlak *et al.* [25].

A plant expression vector incorporating this gene was cloned into the T-DNA segment between the right- and left-border region and introduced into potato via *Agrobacterium tumefaciens*-mediated transfer [22]. The *A. tumefaciens* ABI strain used to deliver this plasmid (pMON10547) contained the disarmed (lacking the T-DNA phytohormone genes) pTiC58 plasmid pMP90RK [16] in the chloramphenicol-resistant derivative of *A. tumefaciens* A208. In this vector the *cryIIIA* gene was driven by the cauliflower mosaic virus 35S promoter with a duplicated enhancer region [15]. The selectable marker and other characteristics of this plasmid have been described [13]. Transformation and regeneration to Russet Bur-

Table 1. Comparison of *cryIIIA* genes. The *cryIIIA* gene improved for plant expression was synthesized by British Biotechnology Products Ltd., Abingdon, England.

	Improved plant wild-type gene	Expression gene
Bases different from wild type	–	399/1791 (22%)
Codons different from wild type	–	347/597 (58%)
G + C content (%)	37	49
Potential polyadenylation sites	24	3
ATTTA sequences	12	0
A + T-rich regions (> 6 consecutive A and/or T)	37	0

bank potato plants was accomplished according to the protocol described [22].

Transformed Russet Burbank potato plants containing the modified *cryIIIA* gene were selected for kanamycin resistance and analyzed for resistance to damage by CPB. A single leaf from a kanamycin-selected plant was incubated for 3 days with 5 newly hatched neonate CPB larvae. A total of 308 transgenic plants were assayed. Fifty-five plants (55 of 308; 18%) that showed complete protection from damage by CPB neonates in an excised leaf bioassay were further characterized. Preliminary experiments demonstrated that plants that showed incomplete protection in this neonate bioassay always contained less than 0.001% CryIIIA protein in their leaf tissue. Because of the sensitivity of neonate CPB to the CryIIIA protein, the bioassay was unable to discriminate among those plants which showed complete protection. Western blot imunoassays were performed on all of the completely protected plants, and these immunoassays [10] showed that completely protected plants expressed the CryIIIA protein as 0.002% to 0.3% of total leaf protein (data not shown). This range and distribution of expression levels is consistent with the level of expression of the modified lepidopteran-specific genes in transgenic tomato, tobacco [25] and cotton [24]. As with other transgenes in plants, this distribution of expression levels in independent transformants has been attributed to position effects and is not correlated with gene copy number or alteration of the gene.

A substantial fraction of the Russet Burbank potato plants (23 of 55) expressed CryIIIA protein at levels of 0.1% of total protein or greater. Compared to potato and tomato plants expressing the wild-type *cryIIIA* gene, which expressed less than 0.001% of total leaf protein, the highest levels of expression of the modified *cryIIIA* gene represent at least a 300-fold increase in plant expression. This dramatic increase in expression via gene modification demonstrates the applicability of this approach for improving *B.t.* gene expression in plants.

The higher levels of *cryIIIA* gene expression correlate with increased protection from CPB. Whole plants were analyzed in growth chamber assays with CPB of different life stages. All plants expressing at least 0.002% CryIIIA protein caused 100% mortality of neonate larvae, conferring complete protection from foliar damage (Fig. 2). When larvae of the less sensitive 2nd and 3rd instar stage were placed on plants expressing higher levels of the CryIIIA protein (0.05% or greater) they rapidly ceased feeding and became moribund. Very little feeding damage was detectable on these plants.

Later larval stages and adult CPB are sensitive to the CryIIIA protein but because of their larger size requiring increased levels of CryIIIA protein during feeding, they are difficult to control in the field with microbial preparations [7]. However, control of adults is important since they give rise to the destructive larval generations and may themselves cause extensive crop damage. CPB overwinter as adults in the soil and begin feeding immediately upon emergence in the spring. We developed a bioassay to determine the effect of the transgenic plants on adults. Excised leaves were exposed to five adult beetles and the extent of feeding damage was determined after 7 days. Leaves from plants that expressed CryIIIA protein at 0.005% or less showed some feeding damage by adult beetles. At levels of expression above 0.005%, feeding by adults was negligible. Although adult beetles did not succumb as quickly as the larval stages, they also ceased feeding on the leaves within 24 h on these plants.

To determine the effects of the transgenic potato plants on oviposition and adult survival, 15 male and 15 female newly emerged adults were placed in cages containing either plants of a highly expressing (0.05% to 0.1% CryIIIA as a percent of total protein) transgenic line or non-transgenic Russet Burbank plants, and the mean numbers of egg masses oviposited in two weeks per cage was measured. On non-transgenic Russet Burbank plants the mean number of egg masses per cage were 117 and 143 in two separate trials. In comparison, beetles fed on the transgenic line oviposited a mean of 1.7 and 0 egg masses per cage in two trials. This decrease in oviposition was further examined by dissection of adult females. The

Fig. 2. A photograph of the results of a whole plant growth chamber CPB bioassay. Whole plant assays performed on plants showing no damage in the excised leaf bioassay, were done by exposing control and *cryIIIA*-expressing plants to 50–100 CPB neonates. Defoliation of control plants was complete in 7–10 days; neonates on the plants with CryIIIA were dead within 48 h.

fat body and ovaries of females fed on the transgenic plants were dramatically reduced in size with developing ova either partially or totally reabsorbed (Fig. 3). The beetles apparently reabsorbed fat body and reproductive tissue as a consequence of their cessation of feeding on the transgenic plants.

Despite the low level of leaf feeding by adults, the CryIIIA insect control protein was at least in part responsible for an accelerated rate of mortality over mere starvation. The lethal time 50 (LT_{50}) as determined for adults on the transgenic plants was measured as 9.7 days. Fifteen days after exposure, 99% of the beetles on the transgenic plants were dead, while only 10% of the beetles which were held without feeding had died.

Field protection of the *cryIIIA*-expressing potato plants from damage by CPB was similar to that observed in the laboratory trials. Twenty-five independent transgenic lines were evaluated in field trials in Wisconsin, Washington, and Oregon. Twenty-three of these lines expressed at least 0.1% CryIIIA protein. Plants were exposed to high levels of natural CPB infestation as the transgenic potato plants were surrounded by border rows of non-transgenic plants in order to attract beetles. The potato plants expressing the *cryIIIA* gene were protected from CPB defoliation over the course of the growing season (Fig. 4) at the three field test sites demonstrating highly effective control of CPB.

The yields of insect protected potatoes were comparable to the yields obtained from control plants treated with insecticides. These new lines were acceptable as Russet Burbank for agronomic and morphological traits such as plant height, branching, leaf morphology and overall vigor. Tuber characteristics such as yield, fry color,

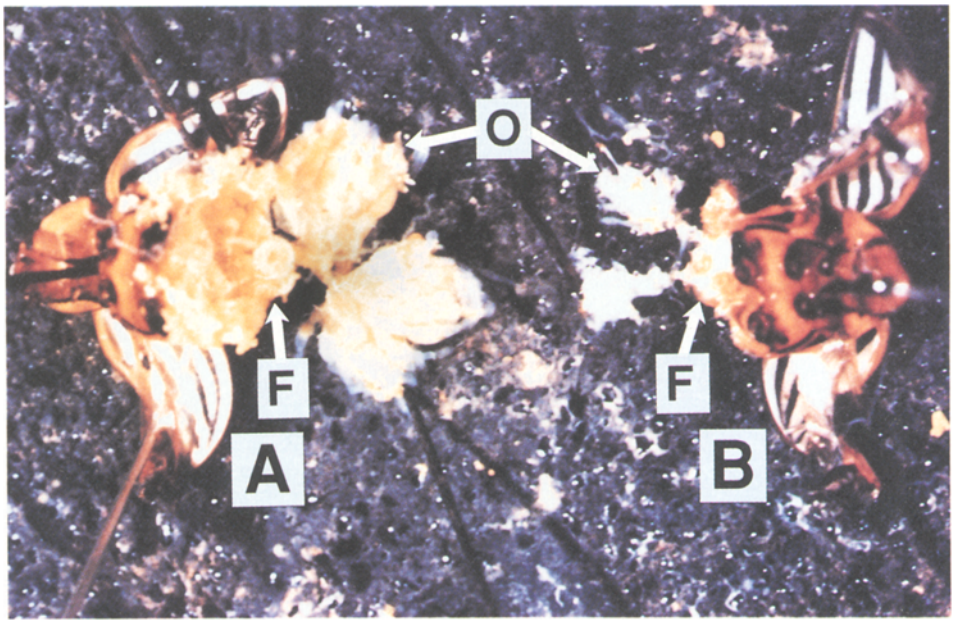

Fig. 3. A photograph of two dissected CPB female adults; the beetle on the left (A) fed on a control plant, and the beetle on the right (B) fed on a plant expressing the *cryIIIA* gene. The females were taken as newly emerged adults and placed in cages with either all control plants or insect resistant plants. The insects were dissected after 10 days of incubation with the plants. Note the size differential in the fat body structures (F) and the ovaries (O).

sugar ends, black spot bruise potential, density and glycoalkaloid content were within established limits for Russet Burbank (Table 2). These and other lines are now in advanced field evaluation and seed certification for eventual commercialization.

Potato plants expressing the modified *cryIIIA* gene were protected from damage by all stages of CPB both in the laboratory and in the field. These protected plants have agronomic and tuber characteristics consistent with standard Russet Burbank potatoes. In recent preliminary taste tests these potatoes compared favorably with control Russet Burbank potatoes.

Based on these results, the plants described in this report represent a new tool for control of Colorado potato beetle without sacrificing any of the advantages of continued utilization of the Russet Burbank variety. The results shown are derived from the first year of field experiments (1991) which focused on demonstrating insect control efficacy. These tests represent only the first step in advanced field evaluation and product development. For these insect-protected potato plants to become an integral part of potato pest management, additional studies of both the plants and the insect populations will be required. The goal of these advanced activities is to develop an integrated pest management (IPM) program that effectively utilizes these genetically modified plants, while at the same time protecting their durability as an insect control system. Field experiments to address these questions have been designed for 1993 and later years.

While the details of an IPM program that incorporates these plants are still in development,

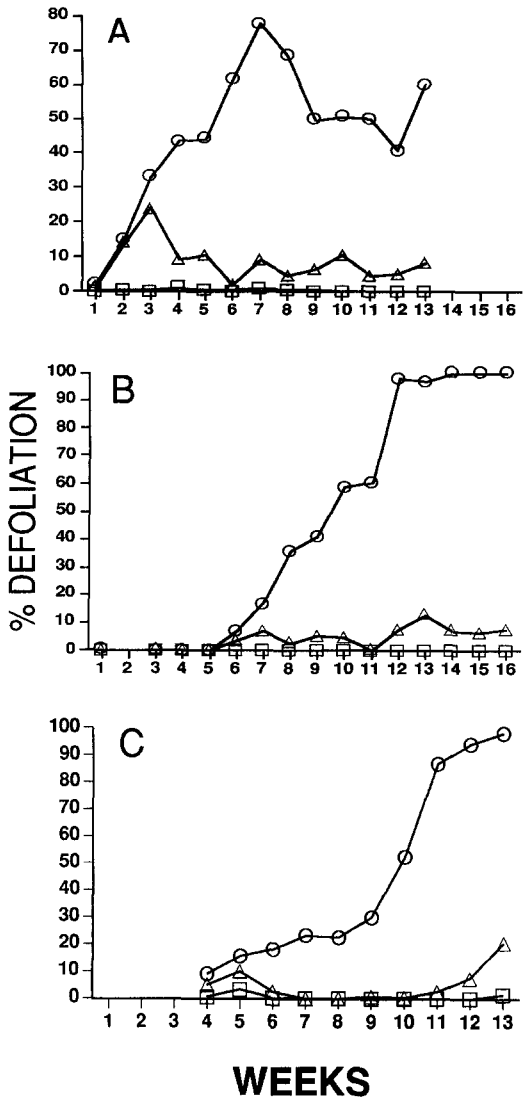

Fig. 4. Results of 1991 field tests in Wisconsin (A), Washington (B), and Oregon (C) comparing % defoliation by CPB of control plants sprayed with chemical insecticides (-△-), control Russet Burbank potato plants without insecticide treatments (-○-), and potato plants expressing the *cryIIIA* gene (-□-). The tests were done with transplanted plantlets. Individual plots, replicated 4 times in a randomized complete block design, consisted of 4 rows (90 cm spacing) by 3 m with each row having 10 plants. Plots were separated by one blank row and one or more rows of control Russet Burbank potatoes. Insecticide treatments varied acording to site. The insecticide treated plots in Wisconsin were sprayed 6 times with Pydrin (113 g active ingredient per ha) in weeks 3, 5, 6, 7, 10 and 11. The treated plots in Oregon were sprayed with Asana (226 g active ingredient per acre) two weeks before the first evaluation of defoliation followed one week later with a granular application of aldicarb at 3375 g active ingredient per ha. In Washington, application of Monitor 4E (1130 g active ingredient per acre) was done on weeks 1, 3, 5, 8 and 15.

Table 2. The rating of selected Monsanto Russet Burbank lines, transformed for insect protection, for processing characteristics (fry color and sugar ends) blackspot potential and glycoalkaloid levels. The controls were untransformed Russet Burbank lines treated as the transformed lines, grown from transplants at each of three locations (Wisconsin, Oregon and Washington). Fry color was rated on a scale of 0–4, a standard USDA grading system, with the lower number indicating lighter color. Sugar-ends are defined as a french fry with one end darker than the remainder of the fry. The dark end must be at least 6.3 mm long and at least one USDA color unit darker than the rest of the fry. The glycoalkaloid analysis [3] and the determination of blackspot potential (abrasive peel method, rated 1–5 with a lower score indicating less blackspot potential [23]) were assessed according to published protocols.

Line	Fry color	Sugar ends (%)	Blackspot	Glycoalkaloids (mg/kg)
Bt6	3.2	40	3.5	161
Bt10	3.5	13	3.2	134
Bt12	2.8	47	3.7	140
Bt16	2.9	33	3.5	129
Bt17	2.7	27	3.5	156
Control	3.0	27	3.1	149

certain properties of these plants provide a number of potential advantages. The high dose of CryIIIA protein in the plants provides a 50- to 100-fold excess over the amount required to control neonate CPB. In addition, we have observed anti-feedant behavior and reduced fecundity of the adults feeding on the plants. Unlike chemical insecticides, these insect protected potato plants have no detrimental effects on hemipteran and spiders, predators which affect CPB and other pest species. These are strong scientific bases to develop an effective, sustainable, IPM program for potatoes. These genetically improved plants will offer an effective alternative for potato pest management in the future.

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