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Expression of *cry*1Ac9 and *cry*9Aa2 genes under a potato light-inducible *Lhca*3 promoter in transgenic potatoes for tuber moth resistance

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Summary

In transgenic potato it is often desirable to couple high-level expression in foliage with no expression in the edible tubers, especially for resistance to pests that primarily infest foliage. To accomplish this we have investigated the use of a light inducible *Lhca*3 promoter for transcriptional control of *cry*1Ac9 and *cry*9Aa2 genes for resistance to potato tuber moth (PTM) (*Phthorimaea operculella*). Thirty-five and thirty-one independently derived transgenic lines of potato cultivar Iwa were regenerated for the *cry*1Ac9 and *cry*9Aa2 genes respectively. Significantly inhibited larval growth of PTM on excised greenhouse-grown leaves was observed in 51% of the *cry*1Ac9-trangenic lines and 84% of the *cry*9Aa2-transgenic lines. RT-PCR analysis identified several transgenic lines with high levels of *cry* gene mRNA in leaves and no to low levels in tubers. Southern and ELISA analyses on eight selected *cry*1Ac9-transgenic lines revealed that they contained 2 to 9 copies of the *cry*1Ac9 gene and the amount of Cry protein in leaves was less than 60 ng g^{-1} of fresh leaf tissue. Southern analysis for four selected *cry*9Aa2-transgenic lines revealed that they contained 2 to 6 copies of the *cry*9Aa2 gene. This study has established that the expression of either the *cry*1Ac9 gene or the *cry*9Aa2 gene in transgenic potato plants offers protection against PTM larval damage in foliage when expressed under the transcriptional control of a *Lhca*3 light-inducible promoter. Several transgenic lines were identified with high *cry* gene expression, high resistance to PTM larvae in the foliage, and no or minimal *cry* gene expression in tubers.

Introduction

Potato tuber moth (PTM) *Phthorimaea operculella* (Zeller) is one of the most damaging insect pests of potato (*Solanum tuberosum* L.) crops in many temperate and tropical countries (Fenemore, 1988). In the field the moths usually lay their eggs on the undersides of leaves or on exposed tubers (Goldson & Emberson, 1985). The larvae, hatching from eggs laid on leaves, cause transparent blotch mines in the foliage. Larvae mine the tubers in the field as well as in storage, reducing tuber quality. The damaged areas can also provide an infection point for pathogens (Plaisted et al., 1994). Various management strategies such as cultural practices, biological and chemical control are used to manage PTM (Goldson & Emberson, 1985; Hanafi, 1999). To contribute an additional component to integrated pest management, the development of transgenic potatoes that express the insecticidal toxins produced by different strains of the soil bacterium, *Bacillus thuringiensis* (Bt), has been investigated (Jansens et al., 1995; Douches et al., 1998; Rico et al., 1998; Cañedo et al., 1999; Li et al., 1999; Chakrabarti et al., 2000; Davidson et al., 2002, 2004).

The Cry1Ac9 toxin (designated under the revised nomenclature of Crickmore et al., 1998), isolated from Bt strain, DSIR732, serotyped as var. *kurstaki*, is known to have insecticidal activity against PTM larvae (Gleave et al., 1992a). The gene encoding this protein was codon-modified to increase its expression in plants and confer resistance to PTM larvae in tobacco (Beuning et al., 2001). The subsequent transfer of this modified *cry*1Ac9 gene under the regulatory control of the CaMV 35S promoter to potato confirmed efficacy against PTM larvae in the main crop in which this pest causes economic damage (Davidson et al., 2002, 2004).

Gleave et al. (1992b) cloned and characterized a *cry* gene, encoding a 129 kDa protein from the Bt strain, DSIR517, serotyped as var. *galleriae*. It has been designated *cry*9Aa2 (Crickmore et al., 1998) and the encoded protein has been shown to have insecticidal activity against PTM larvae (Gleave et al., 1992b). Gleave et al. (1998) modified this *cry*9Aa2 gene into three truncated versions, G7, G10 and G14, with increasing modifications to codon usage. Tobacco plants transgenic for these modified *cry* genes showed resistance to PTM larvae, with higher efficacy associated with more changes to the codon usage (Gleave et al., 1998). The transfer of the G14 version of the *cry*9Aa2 gene under the regulatory control of the 35S promoter to potato provided effective resistance to PTM larvae (Meiyalaghan et al., 2004).

The application of genetic engineering to many food crops, including potato, has raised public concerns, especially when the transferred gene is expressed in the plant components utilised as food (Conner & Jacobs, 1999). The 35S promoter used for transcriptional control of the *cry* genes in previous investigations in potato (Douches et al., 1998; Rico et al., 1998; Cañedo et al., 1999; Li et al., 1999; Chakrabarti et al., 2000; Davidson et al., 2002, 2004) is well known to be constitutively expressed throughout plants (Odell et al., 1985), including potato (Gatehouse et al., 1997). However, it is often desirable to couple high-level expression of the transgene in potato foliage with no expression in tubers. The most effective approach to achieve this involves the isolation of appropriate promoters from potatoes. A good candidate is the potato *Lhca*3.St.1 gene encoding the apoprotein 2 (type III chlorophyll *a*/*b* binding protein) of the light harvesting complex of Photosystem I, isolated from the potato cultivar Bintje (Nap et al., 1993). A 538 bp fragment homologous to this promoter has been isolated from the potato cultivar Iwa. Apart from a 41 bp insertion starting at 281 bp upstream from the putative transcription start site, the DNA sequence shows 96% identity to the DNA sequence of *Lhca*3.St.1 gene from potato cultivar Bintje (Shang et al., 2000).

In this paper we report the independent transfer of modified *cry*1Ac9 and *cry*9Aa2 genes under a light inducible *Lhca*3 promoter into the potato cultivar Iwa. The resulting putatively transformed lines were characterized using multiplex PCR and Southern analysis. The level of transgene expression in leaves and tubers, was determined using multiplex RT-PCR. All putatively transformed lines were also evaluated in a containment greenhouse for plant phenotypic appearance and resistance to PTM larvae in foliage and tubers.

Materials and methods

Vector construction

In order to construct an expression cassette with the Iwa *Lhca*3 promoter (Shang et al., 2000), the 35S promoter of pART7 (Gleave, 1992) was replaced with the Iwa *Lhca*3 promoter. Both the Bluescript plasmid harbouring the Iwa *Lhca*3 promoter and pART7 were digested using *Sac*I and *Xho*I restriction endonucleases. The required fragments were extracted from an agarose gel and ligated together using standard methods (Sambrook et al., 1989). The resulting plasmid, pART7cab, was then cleaved with *Eco*RI and *Bam*HI, and ligated with the 2028 bp coding region of the G14 version of *cry*9Aa2 excised from pART27G14 (Gleave et al., 1998) using *Eco*RI and *Bam*HI. The resulting *Lhca*3-*cry*9Aa2-*ocs*3′ chimeric gene was cloned as a 3.3 kb *Not*I fragment into pART27 (Gleave, 1992) to produce the binary vector pART27cab9Aa2. The 1.9 kb coding region of the *cry*1Ac9 gene was excised from pART27*cry*1Ac9^{B} (Beuning et al., 2001) and cloned as a *Kpn*I and *Hind*III fragment into pART7cab, thereby placing the *cry*1Ac9 gene under transcriptional control of *Lhca*3 promoter. The resulting *Lhca*3-*cry*1Ac9-*ocs*3′ chimeric gene was cloned as a 3.2 kb *Not*I fragment into the binary vector pART27 (Gleave, 1992) to produce pART27cab1Ac9.

The p*nos-nptII-nos*3′ chimeric gene on the binary vectors was used as a selectable marker conferring resistance to kanamycin for the transformation of potato cultivar Iwa. The binary vectors pART27cab1Ac9 and pART27cab9Aa2 (Figure 1) were individually transferred to the disarmed *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) using the

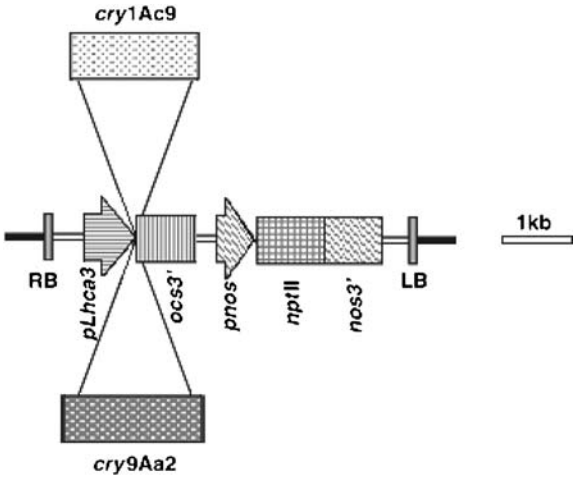

Figure 1. Schematic representation of the T-DNA regions of the two binary vectors (pART27cab1Ac9 and pART27cab9Aa2) used in this study. RB, LB, right and left T-DNA borders respectively.

freeze-thaw method (Höfgen & Wilmitzer, 1988). Prior to co-cultivation with potato tissue, the *Agrobacterium* cultures harboring the binary vectors were cultured overnight at 28 °C, on a shaking table, in LB broth supplemented with 300 mg l^{-1} spectinomycin.

Plant material

Virus-free plants of cultivar Iwa were multiplied *in vitro* on a multiplication medium consisting of MS salts and vitamins (Murashige & Skoog, 1962) plus 30 g l^{-1} sucrose, 40 mg l^{-1} ascorbic acid, 500 mg l^{-1}casein hydrolysate, and 7 g l^{-1} agar. The agar was added after pH was adjusted to 5.8 with 0.1 M KOH and the medium was autoclaved at 121 °C for 15 min. Aliquots of 50 ml were dispensed into pre-sterilised plastic containers (80 mm diameter × 50 mm high; Vertex Plastics, Hamilton, New Zealand). Plants were routinely subcultured as two to three node segments every 3–4 weeks and incubated at 26 °C under cool white fluorescent lamps (80–100 μmol m^{-2} s^{-1}; 16-h photoperiod).

Potato transformation protocol

Fully expanded leaves from the *in vitro* plants were excised, cut in half across midribs, while submerged in the liquid *Agrobacterium* culture. After about 30 s, these leaf segments were then blotted dry on sterile filter paper (Whatman® No. 1, 100 mm diameter). They were then cultured on callus induction medium (multiplication medium supplemented with 0.2 mg l^{-1} napthaleneactic acid and 2 mg l^{-1} benzylaminopurine) in standard plastic Petri dishes (9 cm diameter × 1 cm high) under reduced light intensity (5–10 μmol m^{-2} s^{-1}) by covering the Petri dishes with white paper. After two days, the leaf segments were transferred to the callus induction medium supplemented with 200 mg l^{-1} Timentin™ to prevent *Agrobacterium* overgrowth. Five days later, they were transferred on to the same medium further supplemented with 100 mg l^{-1} kanamycin in order to select the transformed cell colonies. Individual kanamycin-resistant cell colonies (0.5–1 mm diameter), developing on the leaf segments in 3–6 weeks, were excised and transferred on to regeneration medium (potato multiplication medium with sucrose reduced to 5 g l^{-1}, plus 1.0 mg l^{-1} zeatin and 5 mg l^{-1} GA_3, both filter sterilised and added after autoclaving) supplemented with 200 mg l^{-1} Timentin and 50 mg l^{-1} kanamycin in plastic Petri dishes (9 cm diameter × 2 cm high). These were cultured under low light intensity (30–40 μmol m^{-2} s^{-1}) until shoots regenerated. Individual cell colonies, each with regenerated shoots, were transferred to potato multiplication medium containing 100 mg l^{-1} Timentin in plastic containers as described above. Single healthy shoots derived from individual shoot clumps were excised and transferred to multiplication medium containing 100 mg l^{-1} Timentin and 50 mg l^{-1} kanamycin. Individual shoots (one per original shoot clump) that rooted readily in the kanamycin- supplemented medium were labeled and further subcultured on to potato multiplication medium with 100 mg l^{-1} Timentin for micropropagation in plastic containers as described above. All antibiotics were filter sterilised and added, as required, just prior to dispensing the media into culture vessels or dishes.

Screening of putative transformed lines using PCR

Genomic DNA was isolated from *in vitro* shoots of putative transgenic and control plants based on the method described by Bernatzky and Tanksley (1986). DNA was amplified in a polymerase chain reaction (PCR) containing primers specific for the transgene of interest multiplexed with primers for the endogenous potato actin gene as an internal control (Table 1). PCRs were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The reactions included 2.5 μl 10× buffer (750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween® 20), 1.5 μl 25 mM $MgCl_2$, 2.5 μl

Table 1. Primers for PCR of each gene and expected product size

Target gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product size (bp)
*cry*1Ac9	GCCACAGAATAACAACGTGC	GCATACCGTACACGAACTCG	359
*cry*9Aa2	GCACGGAATTATTGGCGCTTC	CACGATGTCCAACACCATCAA	826
*npt*II	ATGACTGGGCACAACAGACAATCGGCTGCT	CGGGTAGCCAACGCTATGTCCTGATAGCGG	612
Actin	GATGGCAGAAGGCGAAGATA	GAGCTGGTCTTTGAAGTCTCG	1069

dNTP (at 2 mM each of dATP, dCTP, dGTP, dTTP), 0.25 μl Red Hot® DNA polymerase at 5 U μl^{-1} (Advanced Biotechnologies, Surrey, U.K.), 0.5 μl of each primer (at 10 μM), 1.0 μl of DNA (10–50 ng) and water to a total volume of 25 μl. The conditions for PCR were: 1 min at 93 °C, followed by 35 cycles of 30 s 92 °C, 30 s 60 °C, 90 s 72 °C, followed by a 6 min extension at 72 °C. Amplified products were separated by electrophoresis in a 2% agarose gel and visualized under UV light after staining with ethidium bromide.

Phenotypic evaluation of transgenic lines in greenhouse

All of the putatively transformed lines were transferred to the containment greenhouse using the method described in Conner et al. (1994). Two plants were established in each of three PB5 bags (15 cm × 15 cm × 15 cm black polythene bags) per line, with each PB5 bag treated as a replicate, and the bags placed in the greenhouse in a randomised block design. The greenhouse conditions provided heating below 15 °C and ventilation above 22 °C. Day length was supplemented to 16 h when needed with 500 W metal halide vapour bulbs, and relative humidity was maintained above 60%.

After 6–8 weeks in the greenhouse, the appearance of the foliage from each line was recorded using the categories: phenotypically normal, marginal leaf curl, leaf wrinkling, reduced vigour, and/or stunted plants (Conner et al., 1994). Tubers were also evaluated based on their size and appearance at the time of harvest, 14 weeks after planting in the greenhouse. Lines that produced tubers <10 mm in length (from apical to distal end) and/or were deformed in shape were considered abnormal. Harvested tubers were immediately transferred into brown paper bags and stored in cool dark storage (temperature 8 °C). In this way exposure to light was minimized to avoid inadvertent induction of transgene expression in the tubers.

Insect bioassay with excised leaves

Young, fully expanded leaves from transgenic and non transgenic control lines grown in the greenhouse for 7–8 weeks were used for the PTM larvae bioassays. The insects used in the bioassays were obtained from a laboratory colony maintained as previously described (Davidson et al., 2002). The petioles of excised leaves were inserted through a polystyrene stopper (2 cm diameter × 1.5 cm long with a full length radial slit) into each 25 ml glass vial full of water. The leaf with glass vial was then placed vertically in a transparent plastic jar (500 ml). One leaf with five leaflets was used per replication and the bioassay was replicated three times per potato line. Five PTM neonate larvae were collectively weighed and placed on the leaflets. Leaf material from each of three replicate plants (which were arranged in a randomized block design on a greenhouse bench) was placed in separate containers. The jar was covered by fine Terylene mesh (pore size approximately – 0.25 mm^2) and placed in a controlled temperature room at 22 ± 3 °C and under a photoperiod of 16 h light and 8 h dark. Surviving larvae were removed after 9 days and weighed individually. A growth index (GI) for each larvae was calculated as GI = $\log_e$(final weight/mean initial weight).

Insect bioassay with tubers

Greenhouse-grown tubers, harvested from transgenic and non-transgenic control lines described above for excised leaf bioassay, were exposed to PTM neonate larvae. For each of three replicates, a 40–60 g whole tuber was punctured approximately 20 times with a 1 mm diameter needle to a depth of 10 mm to facilitate access by the larvae and placed in a 350 ml semi transparent plastic pottle. Ten neonate larvae were placed on to each replicate tuber. The pottle was covered with Terylene mesh (pore size approximately – 0.25 mm^2) and placed in a controlled temperature room at 22 ± 3 °C in darkness. The number

of surviving larvae and pupae were counted after three weeks.

Reverse transcription (RT)-PCR analyses

Total RNA was isolated from leaves and tubers of greenhouse-grown plants using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. For RNA isolation tubers were taken from dark storage and processed in the shortest possible time (less than 10 min) to minimise light exposure. The RNA samples were treated with DNaseI, Amplification Grade (Invitrogen), according to the manufacturer's directions. Following DNaseI treatment, the absence of contaminating DNA in the RNA samples was confirmed by PCR amplification as described above, using the actin gene specific primers which flank two introns. The multiplex RT-PCR was performed on each DNA-free RNA sample using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq (Invitrogen). The RT-PCR primers used for *cry*1Ac9 and actin were the same as those used for PCR analysis (Table 1). For *cry*9Aa2 new primers were designed to conveniently distinguish the RT-PCR product of *cry*9Aa2 from the RT-PCR product of the actin gene. For the *cry*9Aa2 gene the nucleotide sequences of the primers were 5′-GCATCTAATCGCCGTTCA-3′ and 5′-CGAATTTGGTCCGGACTT-3′, which generate an expected PCR product of 424 bp. RT-PCRs were carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The reactions included 1 μl SuperScript™ III RT/Platinum® Taq mix, 12.5 μl 2× Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$), 0.5 μl of each primer (at 10 μM), 1.0 μl of RNA (10–50 ng) and water to a total volume of 25 μl. The conditions for RT-PCR were: 30 min at 55 °C (for cDNA synthesis), 2 min at 94 °C (to denature the SuperScript™ III RT enzyme), 35 cycles of 15 s 94 °C, 30 s 60 °C, 90 s 68 °C (PCR amplification), followed by a 5 min extension at 68 °C. Amplified products were separated by electrophoresis in a 2% agarose gel and visualized under UV light after staining with ethidium bromide.

Southern analysis

Total genomic DNA from young leaves of selected greenhouse-grown plants was extracted based on the method described by Bernatzky & Tanksley (1986). The genomic DNA was digested using *Hin*dIII (NEB, Beverly, MA, USA) at 10 units per μg DNA. *Hin*dIII restricts once and twice within the T-DNA of pART27*cry*1Ac9^{B} and pART27*cry*9Aa2 respectively. Approximately 10 μg of digested DNA was loaded per lane on a 0.8% TAE-buffered agarose gel. Following separation of fragments, the DNA was transferred to Hybond N+ membrane (Amersham, Little Chalfont, UK). Hybridisation was performed with radioactively-labeled DNA probes of *cry*1Ac9 and *cry*9Aa2 consisting of 359 and 826 bp fragments (as PCR products described above) encoding the *cry* genes respectively. Both probes were labelled with [α-^{32}P] dATP using the Megaprime DNA labeling system (Amersham, Little Chalfont, UK). Membranes were placed on Kodak X-Omat XK-1 (blue) X-ray film (Eastman Kodak Company, Rochester, NY) with intensifying screens for 19 days.

Enzyme-linked immunosorbent assay (ELISA)

Due to the unavailability of an ELISA kit to detect *cry*9Aa2 protein, ELISA was performed only for *cry*1Ac9-transgenic lines. A QuantiPlate™ Kit for Cry1Ab/Cry1Ac (EnviroLogix Inc., Portland, ME, USA) was used to detect recombinant protein expression according to the manufacturer's instructions. This kit represents a double-antibody sandwich quantitative ELISA with a detection limit of 1.2 μg l^{-1} for Cry1Ac in sample extracts. Extracts were made from weighed leaf samples, taken from the youngest, fully expanded leaves from six-week-old greenhouse plants. The intensity of colour development was measured spectrophotometrically with a ThermoMax™ microplate reader (Molecular Devices, Sunnyvale, Calif.) at 450 nm. The levels of Cry1Ac protein in the leaf extracts were determined by extrapolation to a standard curve based on sample and standard absorbance values. The OD value of the untransformed Iwa control was subtracted before determining the concentration of Cry1Ac protein.

Statistical analysis

Mean GI for each replicate for each line were analysed with analysis of variance. Percent mortality, pupation and live larvae were analysed with a binomial generalised linear model, with a logit link (McCullagh & Nelder, 1989). Comparisons with the control line were made as part of these analyses, and a probability level of 5% was used throughout to determine significance. Analyses were carried out using GenStat (GenStat Committee, 2003).

Results

Potato transformation

The two T-DNA vectors outlined in Figure 1 were successfully constructed and transformed into potato cultivar Iwa. After 3–4 weeks of placing *Agrobacterium* cocultivated explants on callus induction medium, small cell colonies developed along the cut leaf edges and/or leaf surfaces. Most of the cell colonies were green and grew as hard, compact callus while some were pale-green and friable. Following transfer to regeneration medium, most hard green cell colonies produced shoots, whereas the friable calli generally failed. Two to three weeks after the cell colonies were transferred with regenerated shoots to potato multiplication medium, 2–4 fully grown shoots developed from each original cell colony. In kanamycin selection medium, single healthy shoots excised from each shoot clump readily formed roots within a week. A total of 66 independently derived, putative transgenic potato lines were selected (35 lines for *cry*1Ac9 and 31 lines for *cry*9Aa2), on the basis of their ability to grow and root on kanamycin selection medium.

PCR analysis of regenerated lines

The presence of the *npt*II and *cry* genes in these lines was confirmed using multiplex PCR with an endogenous actin gene as an internal positive control. Since the actin product was expected in both transgenic and non-transgenic potato plants, this allows failed PCR reactions to be conveniently distinguished from a non-transgenic line. PCR products from representative lines are illustrated in Figure 2. All 66 putative transgenic lines were PCR positive for both the *npt*II and *cry* genes.

Greenhouse evaluation and insect bioassays

The majority of the lines were observed to have a phenotypically normal appearance when grown in the greenhouse. Two lines with the *cry*1Ac9 gene (#117 and #128) and three lines with the *cry*9Aa2 gene (#1, #2 and #26) exhibited a range of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigour, abnormally small and/or deformed tubers, or a combination of these traits.

For the population of *cry*1Ac9-transgenic lines, there was substantial variation in larval GI (Figure 3). The larval GI was not significantly less than the non-transgenic control for 17 of the 35 lines. The remaining 18 lines all exhibited a significantly lower larval GI than the control (Figure 3), with a few lines (#107, #116 and #123) severely inhibiting the growth of PTM larvae. The mortality of larvae developing on tubers also varied markedly between the transgenic lines. PTM larval mortality on the control tubers was 13% (95% confidence limits of 6–26%), whereas mortality on the transgenic lines ranged from 3% (95% confidence limits of 0.4–24%) to 53% (95% confidence limits of 33–72%). There was little correlation between mortality on tubers and the larval GI on foliage ($r = -0.21$). Of the 18 transgenic lines that exhibited a significantly lower PTM larval GI than the control on foliage, 11 showed no

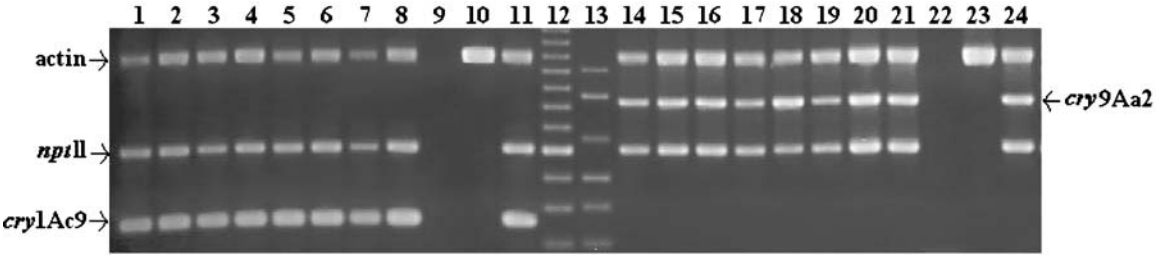

Figure 2. PCR analysis of putative transgenic potato (*Solanum tuberosum*) cv. 'Iwa' lines. Lanes 1–11 represent a multiplex reaction with the *cry*1Ac9 primers producing an expected 359 bp product, the *npt*II primers producing an expected 612 bp product and the actin primers as an internal control producing product 1069 bp. Lanes 14–24 represent a multiplex reaction with *cry*9Aa2 primers producing an expected 826 bp product, the 612 bp *npt*II product and the 1069 bp actin product. Lanes 1–8, lines transformed with pART27cab1Ac9 (#102, #107, #110, #116, #121, #122, #123 and #130 respectively); lanes 9 and 22, no DNA template control; lanes 10 and 23, non-transgenic 'Iwa' control; lane 11, 'Iwa' line I52 known to be transgenic for *npt*II and *cry*1Ac9 (positive control, ex Davidson et al., 2002); lanes 12 and 13, 100 bp molecular ruler 10380-012 and 1 kb plus molecular ruler 10787-018 (Invitrogen, Carlsbad, California) size markers respectively; lanes 14–21, lines transformed with pART27cab9Aa2 (#7, #12, #17, #18, #22, #27, #28 and #29 respectively); lane 24, 'Iwa' line DG4c known to be transgenic for *npt*II and *cry*9Aa2 (positive control, ex Meiyalaghan et al., 2004).

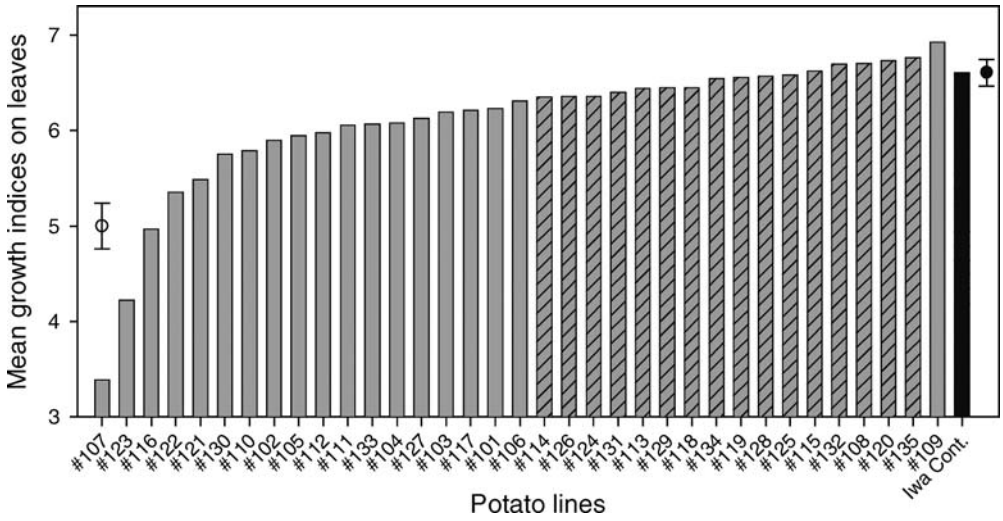

Figure 3. The mean growth indices of potato tuber moth (PTM) larvae reared on leaves of *cry*1Ac9-transgenic potato plants. The error bars represent 95% confidence limits for the mean growth indices for (i) non-transgenic control line (solid circle) (ii) any transgenic line (empty circle). Hatched bars indicate lines that were not significantly different from the non-transgenic control line at 5% level.

significant difference from the non-transgenic control in larval mortality in the tuber bioassays. PTM bioassay results for tubers of the 8 lines with the lowest larval GI on foliage are shown in Table 2. There was little consistency in larval mortality and pupation rates on tubers among these transgenic lines.

For the population of *cry*9Aa2-transgenic lines, foliage from 26 of the 31 lines supported larval growth with significantly lower GI than larvae on foliage from non-transgenic control plants (Figure 4). The larval growth of PTM on 12 of the transgenic lines was severely inhibited (GI under 5.0). Similar to the results with the *cry*1Ac9-transgenic lines, the mortality of larvae developing on tubers varied markedly between the transgenic lines. PTM larval mortality on the control tubers was 12% (95% confidence limits of 5–25%), whereas mortality on the transgenic line ranged from 0 to 43% (95% confidence limits of 0–13% and 25–64% respectively). The percent mortality on the tubers was uncorrelated ($r = 0.04$) with larval GI on leaves. Of the 26 transgenic lines that exhibited a significantly lower PTM larval GI on foliage, 21 showed no significant difference from the non-transgenic control in larval mortality in the tuber bioassays. The PTM bioassay

Table 2. Performance of 8 selected *cry*1Ac9-transgenic lines in bioassays against PTM larvae (95% confidence limits in brackets, df = 75)

Line	Mean GI in leaf bioassay	Larval mortality in tuber bioassay (%)	Individuals remaining as larvae in tuber bioassay (%)	Individuals reaching pupation in tuber bioassay (%)
#107	3.39	23 (10, 44)	70 (49, 85)	7 (2, 25)
#123	4.22	10 (3, 30)	60 (40, 77)	30 (16, 49)
#116	4.97	27 (13, 48)	63 (43, 80)	10 (3, 28)
#122	5.35	33 (17, 54)	63 (43, 80)	3 (0, 22)
#121	5.49	40 (22, 61)	60 (40, 77)	0 (0, 12)
#130	5.75	53 (34, 72)	47 (28, 66)	0 (0,12)
#110	5.79	17 (6,37)	60 (40, 77)	23 (11, 43)
#102	5.90	43 (25, 64)	43 (25, 63)	13 (5, 32)
Control	6.61 (±0.20)	13 (6, 26)	37 (24, 51)	50 (37, 63)

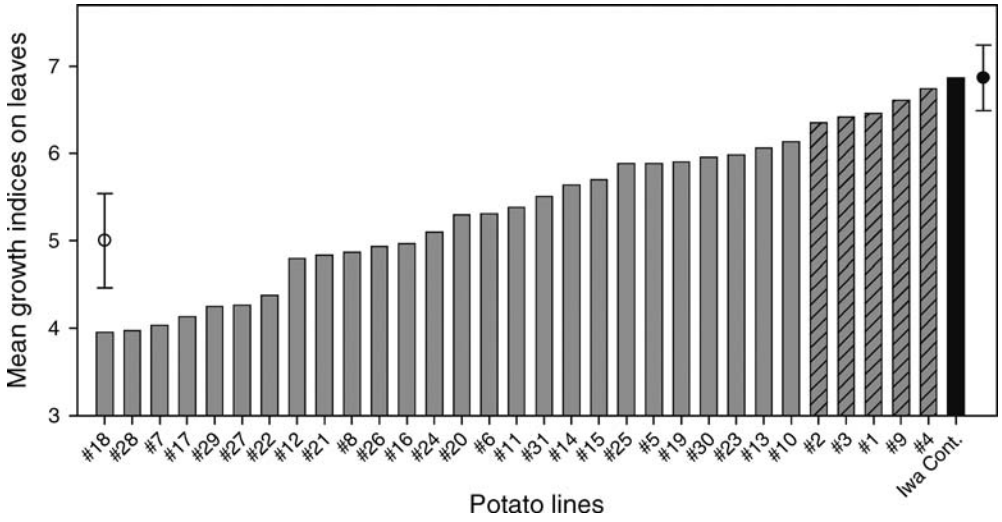

Figure 4. The mean growth indices of potato tuber moth (PTM) larvae reared on leaves of *cry*9Aa2-transgenic potato plants. The error bars represent 95% confidence limits for the mean growth indices for (i) non-transgenic control line (solid circle) (ii) any transgenic line (empty circle). Hatched bars indicate lines that were not significantly different from the non-transgenic control line at 5% level.

results for the 8 lines with the lowest larval GI on foliage are shown in Table 3. Amongst these lines, there was little consistency in mortality and pupation rates on tubers. Mortality on tubers of these selected lines was reasonably similar to the control tubers, but pupation was significantly lower than the control except for the transgenic lines #7, #12 and #17.

RT-PCR analysis of selected transgenic lines

The expression of the *cry* genes in leaves and tubers of transgenic lines was determined by multiplex RT-PCR analysis with an endogenous actin gene (Figure 5). The use of the endogenous actin gene as an internal control allows failed reactions to be conveniently distinguished from non-expressing transgenic line and also provides a baseline for standardising gene expression between transgenic lines. Since the primers flank two introns in the actin gene, it also provides a convenient check for DNA contamination of the RNA samples. RT-PCR analysis using actin gene primers produced the expected 709 bp product in all samples. However, RT-PCR actin products also showed a faint 835 bp fragment in some samples.

Table 3. Performance of 8 selected *cry*9Aa2-transgenic lines in bioassays against PTM larvae (95% confidence limits in brackets, df = 67)

Line	Mean GI in leaf bioassay	Larval mortality in tuber bioassay (%)	Individuals remaining as larvae in tuber bioassay (%)	Individuals reaching pupation in tuber bioassay (%)
#18	3.95	20 (8, 41)	47 (30, 65)	33 (18, 52)
#28	3.98	10 (3, 30)	67 (48, 81)	23 (11, 42)
#7	4.03	13 (4, 34)	23 (11, 42)	63 (44, 79)
#17	4.13	10 (3, 30)	10 (3, 27)	80 (61, 91)
#29	4.25	10 (3, 30)	63 (45, 79)	27 (14, 46)
#27	4.27	13 (4, 34)	67 (48, 81)	20 (9, 39)
#22	4.38	13 (4, 34)	60 (42, 76)	27 (14, 46)
#12	4.79	10 (3, 30)	7 (2, 24)	83 (65, 93)
Control	6.87 (±0.46)	12 (5, 25)	13 (7, 25)	75 (62, 85)

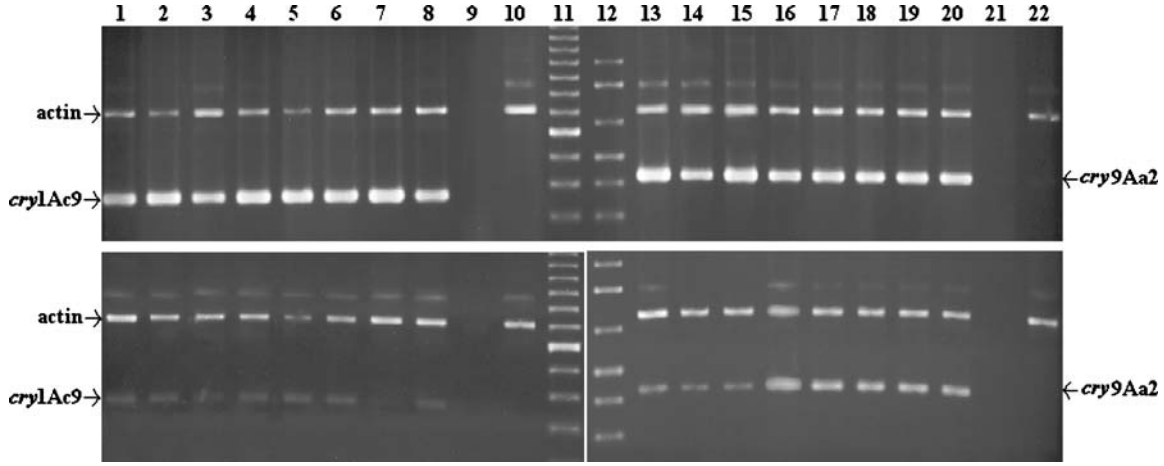

Figure 5. Reverse transcription (RT)-PCR analysis of transgenic potato lines. Upper panel represents RT-PCR analysis of RNA isolated from leaves and lower panel represents RT-PCR analysis of RNA isolated from tubers. Lanes 1–10 represent a multiplex reaction with the *cry*1Ac9 primers producing an expected 359 bp product and the actin primers as an internal control producing an expected 709 bp product. Lanes 13–22 represent a multiplex reaction with *cry*9Aa2 primers producing an expected 424 bp product and the 709 bp actin product. Lanes 1–8, *cry*1Ac9-transgenic lines #102, #107, #110, #116, #121, #122, #123 and #130 respectively; lanes 9 and 21, no RNA template control; lanes 10 and 22, non-transgenic 'Iwa' control; lanes 11 and 12, 100 bp molecular ruler 10380-012 and 1 kb plus molecular ruler 10787-018 (Invitrogen, Carlsbad, California) size markers respectively; lanes 13–20, *cry*9Aa2-transgenic lines #7, #12, #17, #18, #22, #27, #28 and #29 respectively.

RT-PCR showed that amplification of the expected 359 and 424 bp fragments had occurred in all the transgenic lines for the *cry*1Ac9 and *cry*9Aa2 genes respectively. These were substantially brighter bands than for the expression of the actin gene in leaf samples and considerably fainter than the actin bands for tuber samples from most transgenic lines in both *cry*1Ac9- and *cry*9Aa2-transgenic lines (Figure 5).

Southern analysis and ELISA

The transgenic status of eight high performing *cry*1Ac9-transgenic lines and four high performing *cry*9Aa2-transgenic lines was further confirmed by Southern analysis. When restricting the DNA with *Hin*dIII and probing with the *cry* gene, a single band greater than 2.9 and 2.1 kb is expected for each intact insertion of the *cry*1Ac9 and *cry*9Aa2 respectively. Two to nine copies of the *cry*1Ac9 gene and two to six copies of the *cry* 9Aa2 gene were evident in the independently derived transgenic lines (Figure 6).

The amount of Cry protein expressed by the *cry*1Ac9 transgenic potato lines was less than 60 ng per gram of fresh leaf tissue, which represents the detection limit of the ELISA method used.

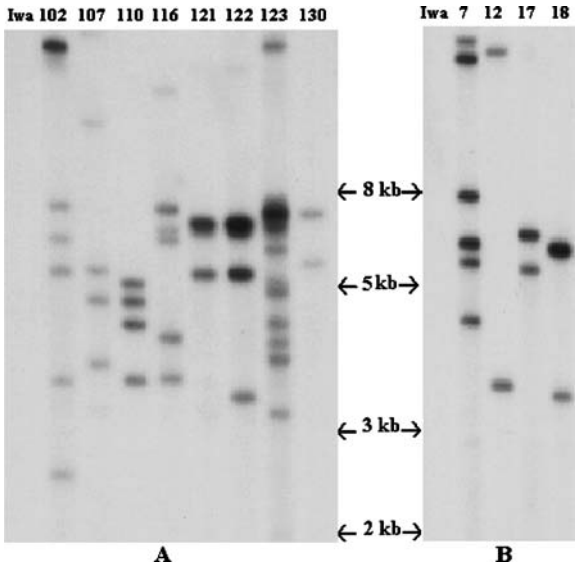

Figure 6. Southern analysis of selected transgenic potato lines (labels above the lanes refer to the plant lines analysed). (A) *cry*1Ac9-transgenic lines. Probe used for hybridization was a 359 bp DNA fragment corresponding to the 3′ end of the *cry*1Ac9 sequence. DNA was restricted with *Hin*dIII. A single band greater that 2.9 kb is expected for each intact insertion of the *cry*1Ac9 gene. (B) *cry*9Aa2-transgenic lines. Probe used for hybridization was a 826 bp DNA fragment corresponding to the 5′ end of the *cry*9Aa2 sequence. DNA was restricted with *Hin*dIII. A single band greater that 2.2 kb is expected for each intact insertion of the *cry*9Aa2 gene.

Discussion

The development of transgenic plants to confer insect pest resistance is becoming a valuable component of integrated pest management (IPM) programmes. However, the application of genetic engineering to many food crops, including potato, has raised public concerns (Conner & Jacobs, 1999). In potato, it is often desirable to couple high-level expression of the transgene in foliage with no expression in tubers. Constitutive promoters have been used to drive *cry* gene expression in all previous studies targeting PTM resistance in transgenic potatoes (Douches et al., 1998, 2002; Jansens et al., 1995; Cañedo et al., 1999; Li et al., 1999; Chakrabarti et al., 2000; Mohammed et al., 2000; Davidson et al., 2002, 2004). Since PTM generally infests foliage of potato crops prior to the tubers (Foot, 1979), preventing foliar infestation may be sufficient to avoid tuber infestation. Therefore, the use of a foliage-specific promoter for transcriptional control of transgenes targeting PTM in potatoes may provide a valuable component for IPM, while simultaneously avoiding transgene expression in tubers to help allay some public concerns associated with the transgenic technology in potato crops.

This study has investigated the value of the potato *Lhca*3 promoter, known to be light-inducible (Nap et al., 1993; Shang et al., 2000), as a potential foliage-specific promoter for the transcriptional control of *cry* gene expression in potatoes. Analysis of all putative transgenic lines using PCR established the presence of the *npt*II and either *cry*1Ac9 or *cry*9Aa2 genes in all regenerated lines (e.g. Figure 2), thereby confirming their transgenic status and a high rate of success for the *Agrobacterium*-mediated gene transfer system of potato using kanamycin resistance as a selectable marker (Barrell et al., 2002).

Neonate larvae are most susceptible to Cry proteins (Roush, 1996) and PTM are usually in this life stage when first encountering potato tissue (Fenemore, 1988). For these reasons, neonate PTM larvae were used in the bioassays. The bioassay method used was based on the assumption that the level of Cry protein in excised leaves was similar to that in the foliage of intact plants. The same assumption has been made in similar studies involving PTM bioassays (Ebora et al., 1994; Jansens et al., 1995; Westedt et al., 1998; Beuning et al., 2001; Douches et al., 1998, 2002; Li et al., 1999; Davidson et al., 2002, 2004), and has been validated by the observed high correlation between excised leaves and intact plants for growth of PTM larvae in response to *cry* gene expression (Davidson et al., 2002). In the case of the leaf bioassays, the final larval weights were made toward the end of the exponential growth phase at day 9 and were therefore appropriate for the calculation of a growth rate. After 9 days the rate of larval weight gain starts to diminish as they begin to lay down fatty tissue in preparation for pupation (Beuning et al., 2001). In the case of the tuber bioassays, the numbers of individuals in the larval and pupal stages were recorded after three weeks. The earlier recovery of larvae for weighing was not possible due to difficulties in finding and removing undamaged larvae from the tubers.

From the two populations of independently derived transgenic lines, 16% of the *cry*9Aa2-transgenic lines and 49% of the *cry*1Ac9-transgenic lines failed to exhibit improved resistance to PTM larvae in the bioassay with excised leaves (Figures 3 and 4). This may be due to insufficient expression or accumulation of Cry protein in the foliage of these transgenic lines to inhibit growth of PTM larvae. The remaining transgenic lines showed significantly lower GI than the non-transgenic control in the PTM bioassays with excised leaves. Larvae recovered from these lines after the 9-day bioassay were small and of substantially lower weight. In our previous studies, PTM larvae with such poor growth in response to *cry* gene expression in transgenic potatoes have failed to reach pupation, resulting in complete disruption to the life cycle (Davidson et al., 2002).

The majority of the lines that gave good resistance to PTM larvae in foliage did not show significantly higher mortality on tubers than the non-transgenic control tubers (Tables 2 and 3). However, at the end of the 3-week tuber bioassay, some transgenic lines had higher percentages of larvae and lower levels of pupation than non-transgenic control tubers. This indicates that the *cry*1Ac9 or *cry*9Aa2 gene expression present in tubers of some transgenic lines may be sufficient to slightly slow the development of the PTM larvae. However, lines #110 and #123 with the *cry*1Ac9 gene and lines #7, #12 and #17 with the *cry*9Aa2 gene showed the low larval mortality and high frequency of pupation similar to that of the non transgenic controls. These lines also exhibited high resistance to PTM larvae in foliage. The difference in insecticidal activity between foliage and tubers of these lines can be attributed to a higher level of *cry* gene expression in foliage and minimal and/or no expression in tubers due to the light-inducible *Lhca*3 promoter (Figure 5).

Considerable variation in the level of PTM resistance among the independently derived transgenic potato lines was observed in this study (Figures 3 and

4). Similar variability in transgene expression among insect-resistant transgenic plants has been commonly reported in other studies (e.g. Peferoen et al., 1990; Van Rie et al., 1994; Beuning et al., 2001), including Iwa potato lines transgenic for either the *cry*1Ac9 gene (Davidson et al., 2002, 2004) or the *cry*9Aa2 gene (Meiyalaghan et al., 2004) under the control of CaMV 35S promoter. Such variation is usually attributed to unpredictable levels of transgene expression as a consequence of position effects resulting from differences in the integration site of the transgenes within the plant genome (Peach & Velten, 1991; Conner & Christey, 1994) and/or differences in T-DNA copy number (Hobbs et al., 1993). Such variation in transgene expression is therefore not promoter dependent.

The sequence *StPoAc58* (GenBank accession X55749) was used to design primers for the potato actin gene. Based on this sequence the primers are predicted to produce a 1041 bp PCR product, including two introns. However, the actin primers produced a 1069 bp PCR product for the potato cultivar Iwa (Figure 2). The DNA sequence of this product shows 95% identity to the actin *StPoAc58* sequence. Based on the *StPoAc58* sequence, the 1069 bp fragment of the Iwa actin gene is predicted to contain two introns of 234 and 126 bp respectively. In RT-PCR analysis the actin primers produced the expected 709 bp product as a bright band (Figure 5), which is equivalent to the genomic fragment minus the two introns. However, sometimes a 835 bp product was also observed as a faint band (Figure 5). The latter RT-PCR product is assumed to have retained the 126 bp intron. A reverse primer designed in this intron produced the predicted product size in RT-PCR analysis (data not shown), therefore confirming the presence of an incompletely spliced mRNA in the RNA samples.

RT-PCR analysis of leaf RNA of selected lines confirmed the high level of transcriptional expression of the *cry*1Ac9 or *cry*9Aa2 genes in the foliage of transgenic plants, as indicated by the higher brightness of the expected products of the *cry* genes relative to the actin gene (Figure 5). This high *cry* gene expression in foliage can be attributed to the *Lhca*3 promoter. Other studies have also reported that the *Lhca*3.St.1 promoter gave higher expression in the foliage of transgenic chrysanthemum (Annadana et al., 2001), potato and tobacco (Nap et al., 1993) plants.

RT-PCR analysis of RNA from tubers revealed that a faint band of *cry* mRNA was detectable in the transgenic tubers containing either the *cry*1Ac9 or *cry*9Aa2 gene. Therefore, transcriptional expression of the *cry* gene by the *Lhca*3 promoter was relatively low in tubers. Furthermore, the level of *cry* mRNA was substantially lower than the mRNA of endogenous potato actin gene in tubers as judged by the relative brightness of the expected products from tuber RNA. This suggests that the high expression of the *cry* genes in foliage coupled with their minimal expression in tubers can be attributed to the *Lhca*3 promoter. These results are supported by the PTM bioassay data which established a marked difference between the phenotypic expression of insect resistance in the foliage and tubers of the transgenic potato lines.

Previously, northern analyses confirmed that the *Lhca3*.St.1 gene was expressed predominantly in foliage and not expressed in tubers of potato (Nap et al., 1993). In contrast, the occasional expression of *cry* genes in tubers, observed in this study, could be due to position effects as a consequence of random integration of the transgene into the potato genome (Conner & Christey, 1994). The very low level of *cry* gene expression observed in tubers of transgenic lines using RT-PCR is possibly a consequence of brief exposure to light at the time of harvest or during RNA isolation.

The majority of the independently derived transgenic lines with either the *cry*1Ac9 gene or the *cry*9Aa2 gene were observed to have a phenotypically normal appearance when grown in the greenhouse. Two *cry*1Ac9-transgenic lines and three *cry*9Aa2-transgenic lines exhibited a range of off-type characteristics such as marginal leaf curl, leaf wrinkling, reduced vigour, abnormally small and/or deformed tubers, or a combination of these traits. Such abnormal phenotypes are frequently observed among transgenic plants and are attributed to somaclonal variation that arises during the cell culture phase of plant transformation (Conner & Christey, 1994).

In contrast to the observed high *cry*1Ac9 transcriptional expression in leaves (Figure 5), the level of Cry protein remained under the detection limit of the ELISA method used (60 ng g^{-1} fresh weight of leaf tissue). A similar result was obtained in previous studies with CaMV 35S promoter (Davidson et al., 2002, 2004). Although the *cry*1Ac9 gene was estimated to range from 2 to 9 copies per genome based on Southern analysis, the Cry protein level was not detectable by ELISA. However, our results do indicate that the low level of Cry1Ac9 protein expressed in the foliage is sufficient to dramatically inhibit PTM larval growth rate to a level known to break the PTM life cycle and prevent pupation.

This study has established that the expression of either the *cry*1Ac9 or *cry*9Aa2 gene in transgenic potato plants offers protection against PTM larval damage in foliage when expressed under the transcriptional control of the light-inducible *Lhca*3 promoter. Several transgenic lines were identified with high resistance to PTM larvae in the foliage and with no or minimal *cry* gene expression in tubers.

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