J. Amer. Soc. Hort. Sci. 124(3):218–223. 1999.

Coexpression of Potato PVY^o Coat Protein and *cryV-Bt* Genes in Potato

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Additional index words. cotransformation, potato tuber moth, potato virus Y^o (PVY^o), virus coat protein, *Bacillus thuringiensis*

Abstract. The codon-modified *cryV-Bt* gene (*cryV-Bt*) from *Bacillus thuringiensis* subsp. *kurstaki* Berliner, which is specifically toxic to Lepidoptera and Coleoptera insects, and a potato virus Y^o coat protein gene (*PVY^{o}cp*), in which the aphid transmission site was inactivated, were cotransformed into potato (*Solanum tuberosum* L.) 'Spunta' via *Agrobacterium tumefaciens* Conn. We demonstrated the integration and expression of both genes by molecular analysis and bioassays. All *cryV-Bt*/*PVY^{o}cp*-transgenic lines were more resistant to potato tuber moth (*Phthorimaea operculella* Zeller) and PVY^o infection than nontransgenic 'Spunta'. Four *cryV-Bt*/*PVY^{o}cp* transgenic lines were equal in potato tuber moth mortality to a *cryV-Bt* transgenic line, but of these four only two lines were equivalent in PVY^o titer levels to a *PVY^{o}cp*-transgenic line. We identified two transgenic lines, 6a-3 and 6a-5, which showed greater resistance to potato tuber moth and PVY^o than the other *cryV-Bt*/*PVY^{o}cp* transgenic lines. This study indicated that multiple genes, conferring insect pest resistance and virus resistance, could be engineered into and expressed simultaneously in a potato cultivar.

Potato tuber moth (*Phthorimaea operculella*) and potato virus Y^o (PVY), a member of the Potyviridae family, both cause severe damage to the cultivated potato (*Solanum tuberosum*). Yield losses of 30% to 70% as well as damage and rotting in storage can result from tuber moth infestation and a yield depression of up to 80% can result from PVY depending on the viral strain and the potato variety (Raman and Palacios, 1982). In addition to the application of agricultural chemicals, genetic changes in the potato plant by traditional breeding have been used for many years to reduce the damage of potato tuber moth and PVY (Ross, 1986). However, a limited source of genes conferring resistance to diseases and insects exists in potato germplasm. Due to the tetraploid ($2n = 4x = 48$) nature of potato, improvement by breeding is inefficient compared to that of diploid species. Thus, the introgression of natural resistance genes from potato germplasm for combined control of PVY disease and potato tuber moth has been a challenge for potato breeders.

Genetic engineering now provides an alternative tool to control PVY and potato tuber moth via insertion of resistance genes into agronomically useful cultivars. This approach has the advantage to preserve the desirable traits of the recipient cultivar, which could otherwise be lost through breeding and selection (Lawson et al., 1990). The *cryI* and *cryV* insecticidal crystal protein genes of *Bacillus thuringiensis* subsp. *kurstaki* Berliner (Bt) bacteria have been cloned, sequenced, modified, and transformed into potato cultivars. These transgenic cultivars expressed a high level of resistance to potato tuber moth (Van Rie et al., 1994; Douches et al., 1998). Since the initial demonstration of coat-protein (cp) mediated resistance against tobacco mosaic virus infection in transgenic tobacco (Powell-Albel et al., 1986), several *PVY^{o}cp* genes have been isolated from viruses and introduced into potato cultivars through *Agrobacterium tumefaciens* Conn-mediated transfer. The expression of viral coat protein genes in transgenic potato is very effective in protecting agronomically important potatoes against PVY (Lawson et al., 1990; Malnoe et al., 1994). Cotransformation of pest resistance and virus resistance has been demonstrated in tobacco (Liang et al., 1994) with promising results.

In an attempt to provide resistance to potato tuber moth and PVY in a major potato cultivar through genetic engineering, a codon-modified *cryV-Bt* gene [now designated *cry1Ia1* under revised nomenclature (Crickmore et al., 1995)] and a *PVY^{o}cp* gene were cloned into the same vector. This construct formed two independent gene expression cassettes, each regulated by their own CaMV 35S promoter (Fig. 1). The purpose of this research was to determine whether a multiple gene construct would effectively express both genes by conferring resistance to potato tuber moth and PVY at a level equivalent to transgenic lines expressing these genes independently. We also report on the greenhouse and laboratory bioassays to evaluate PVY and potato tuber moth resistance, respectively.

Materials and Methods

Agrobacterium transformation vector

Agrobacterium tumefaciens strain LBA4404 (Ooms et al., 1982) was used in the transformation experiments. The PVY^{o}cp gene, which is harbored in the pBI121 vector (Clontech, Palo Alto, Calif.) and modified to inactivate the aphid transmission site, was obtained from T. German, University of Wisconsin,

Received for publication 7 Aug. 1998. Accepted for publication 4 Feb. 1999. We thank Garst Seed/Zeneca Inc. for providing the codon-modified *cryV-Bt* gene. This work was supported in part by grant 263-0152-A-00-3036-00 from a USAID/CIRO/AGR/A program and the Michigan Agricultural Experiment Station. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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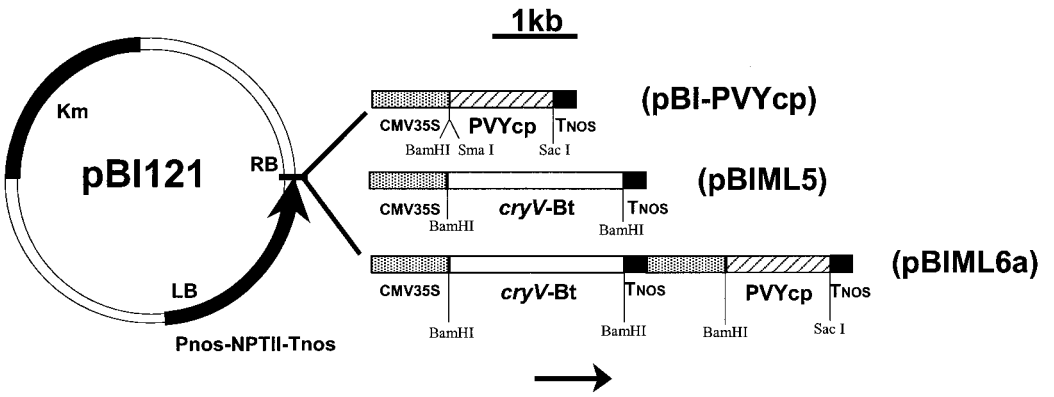

Fig. 1. Schematic figure of the plant expression vectors pBI-PVY, pBIML5, and pBIML6a used to express the *PVY°cp* and *cryV-Bt* gene proteins in transgenic potato plants.

Madison. The plasmid pBI121 was modified to remove the β-glucuronidase (GUS) gene. A codon-modified (J. Tippett, Garst Seeds, Slater, Iowa, personal communication) version of the *cryV-Bt* gene (2.2 kb) (Tailor et al., 1992) was obtained from Garst Seeds/Zeneca (Berkshire, U.K.) and was inserted into the modified pBI121 plasmid to yield pBIML5. The pBIML5 vector was further modified by digesting with EcoRI (a site downstream of the NOS terminator) and blunt-ending by Klenow treatment. The linearized expression cassette of *PVY°cp* gene including a CaMV 35S promoter digested from pBI-*PVY°cp*, was cloned into pBIML5 to create the 13kb vector PBIML6a (Fig. 1). The plasmids pBIML6a, pBIML5, and pBI-*PVY°cp* were mobilized into *A. tumefaciens* LBA4404 from *E. coli* by triparental mating (Bevan et al., 1984).

Production of transgenic plants

'Spunta' potato plants were micropropagated in GA-7 Magenta vessels each containing 25 mL of modified MS basal medium (Douches et al., 1998). The plants were grown at 23 to 27 °C in a 16 h photoperiod under fluorescent lights (30 μmol·m^{-2}·s^{-1}) for 2 weeks. The leaves, with tip and petiole ends removed, were then cultured abaxial side down on agar solidified step I medium (Yadav and Sticklen, 1995) for 2 to 4 d. The precultured explants were immersed for 5 to 10 min at room temperature in an *A. tumefaciens* LBA4404 (pBIML6a or pBI-PVY) culture, which had been grown first in liquid Ty medium (Douches et al., 1998) at 28 °C for 2 d, diluted 10-fold, and then incubated for 6 h. Following immersion, the explants were transferred onto agar-solidified step I medium and cocultured for 2 d at 23 to 27 °C. After the cocultivation, leaf explants were rinsed with liquid step II medium (Douches et al., 1998) supplemented with 200 mg·L^{-1} Timentin (SmithKline Beecham, Philadelphia, Pa.). The explants were then transferred to agar solidified step II medium, supplemented with 200 mg·L^{-1} Timentin and 50 mg·L^{-1} kanamycin. The leaf explants were subcultured every 2 weeks. Regenerating shoots (>5 mm) from separate transformation events were excised and transferred individually into 25 × 100 mm tubes each containing 20 mL of MS medium (Douches et al., 1998) supplemented with 200 mg·L^{-1} Timentin, and 50 mg·L^{-1} kanamycin. PCR-positive rooted plantlets were subcultured and subsequently transplanted into Baccto potting medium (Michigan Peat Co. Houston, Texas) in the greenhouse. The leaves and tubers from these plants were used as tissues for the molecular expression assays.

Detection of *PVY°cp* gene and *cryV-Bt* gene integration and expression

PCR amplification. Genomic DNA was extracted from leaf tissue according to Saghai-Maroof et al. (1984). The primers used for amplification of a 0.6 kb fragment from the *cryV-Bt* gene were as follows: upstream 5'-AGC AGC CAG TCC CTT CCC GCT TCA G-3' and downstream 5'-GGA CCA TCG GCG GCA CCC TCA ACA T-3'. The primers used for amplification of a 0.5 kb fragment from the *PVY°cp* gene were as follows: upstream 5'-CTC GGG CAA CTC AAT CAC AGT TT-3' and downstream 5'-GAA CAC AGA GAG GCA CAC CAC CGA-3'.

Southern analysis. The *cryV-Bt*/*PVY°cp*-transgenic plants, nontransgenic cultivars, a *cryV-Bt* transgenic 'Spunta' (G3), and a *PVY°cp*-transgenic 'Spunta' pBI-*PVY°cp* (Y1) were evaluated by Southern analyses. Genomic DNA (20 μg) was digested with *Bam*HI to excise the *cryV-Bt* gene for hybridization to the *cryV-Bt* gene, and *Bam*HI and *Sac*I were used to excise the 1.2 kb *PVY°cp* gene for hybridization with the *PVY°cp* gene. Genomic DNA (20 μg) which was digested with *Bam*HI and hybridized with the *PVY°cp* gene was used to analyze copy number. The fragments were electrophoretically separated through a 1% agarose gel and transferred onto a nylon membrane (Hybond N, Amersham, U.K.). Prehybridization was conducted for 2 h at 42 °C and hybridization was performed at 42 °C overnight in fresh hybridization solution (Douches et al., 1998) that contained a probe which was random primed labeled using DIG-11-dUTP according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.). Following hybridization, the membrane was washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature and then twice in 0.5× SSC, 0.1% SDS for 20 min at 65 °C. For chemiluminescence detection, disodium 3-(4-methoxyspiro{1,2-dioxetance-3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}] decan}-4-yl) phenly phosphate (CSPD) was used according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.) and the membrane was exposed to X-ray film (Hyperfilm, Amersham) for 15 to 30 min.

Northern analysis. The *cryVBt*/*PVY°cp*-transgenic plants, nontransgenic cultivars, two *cryV-Bt* transgenic 'Spunta' lines (G2, G3), and a *PVY°cp*-transgenic 'Atlantic' pBI-PVY(Y1) were evaluated by northern analysis. Total RNA from young leaf and tuber tissues from greenhouse-grown plants were isolated using Qiagen RNeasy Plant Total RNA Kit (Qiagen, Chatsworth, Calif.). RNA (20 μg) along with ethidium bromide was fractionated by formaldehyde gel electrophoresis through a 1.0% agarose gel in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7). Equal loading was observed by examining the ethidium bromide stained gel with an ultraviolet light. The gel was then blotted onto a Hybond N nylon membrane. Prehybridization, hybridization, and detection conditions were as described above for DNA analysis, except the membrane was hybridized at 52 °C and the *cryV-Bt* and the *PVY°cp* gene probes were synthesized by *in vitro* RNA transcription using an RNA labeling kit (BMB, Indianapolis, Ind.).

Western analysis. Five *cryV-Bt*/*PVY°cp* transgenic lines, nontransgenic 'Spunta' and USDA8380-1 and two *cryV-Bt* transgenic lines (G2, G3) were compared in western analysis. Total protein was isolated from fresh leaf tissue (0.2 g) by grinding it in 800 mL of

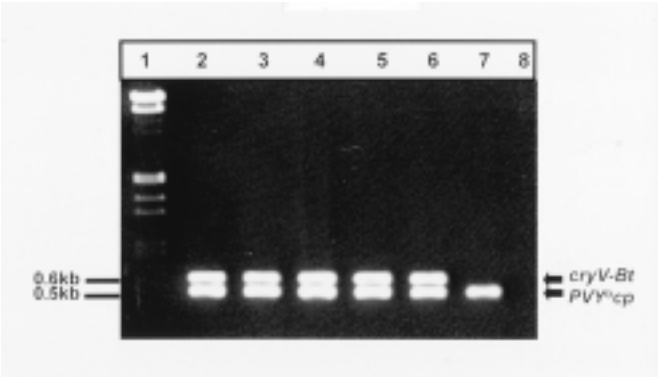

Fig. 2. PCR amplification for the *PVY°cp* and *cryV-Bt* genes. Lane 1 = l DNA ladder digested by *Hind*III and *Eco*RI; lanes 2 to 6 = 6a-1 to 6a-5 (transgenic plants with pBIML6a construct); lane 7 = Y-1 (transgenic plant with *PVY°cp*); lane 8 = Spunta (nontransgenic plant).

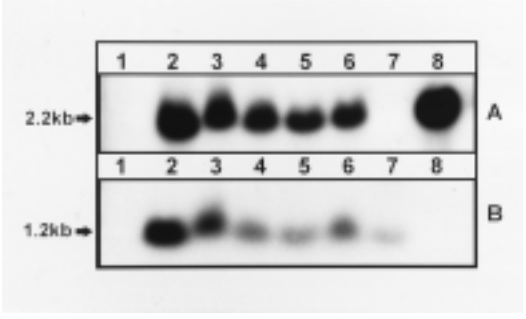

Fig. 3. Southern analysis. (**A**) Genomic DNA digested with *Bam*HI and hybridized with the *cryV-Bt* gene probe. (**B**). Genomic DNA digested with *Bam*HI and *Sac*I and subsequent hybridization with the *PVY°cp* gene probe. Lane 1 = Spunta (nontransgenic plant); lanes 2 to 6 = 6a-1 to 6a-5; lane 7 = Y-1 (transgenic plant with *PVY°cp* gene only); lane 8 = G-3 (transgenic plant with *cryV-Bt* only).

protein extraction buffer (50 mM Tris HCl pH 8.0, 1 mM EDTA, 10 mM diethyldithiocarbamic acid, 0.05% Tween 20). A Bradford protein assay (BioRad, Hercules, Calif.) was conducted on the soluble leaf extracts. For each sample, 150 mg of total protein was loaded onto a 10% SDS-PAGE gel. Identical samples were loaded onto a second gel and both gels were subjected to electrophoresis at 45 v and run overnight. One of the gels was then stained with Coomassie Brilliant Blue R-250 solution to visually compare loading. The other gel was electroblotted to an Immobilon-P (Millipore, Bedford, Mass.) membrane in transfer buffer (25 mM Tris, 192 mM glycine, and 15% methanol). Following transfer, the membrane was blocked with 5% skim milk in TBST (20 mM Tris HCl pH 8.0, 250 mM NaCl, 0.05% Tween 20) for 30 min, and then probed with a rabbit polyclonal *cryV-Bt* antibody (provided by Dilip Dias, Garst Seeds, Slater, Iowa) at 1:60,000 dilution. The purified *cryV-Bt* protein was used as a standard along with Protein Molecular Weight Standards, High Range (Gibco BRL, Gaithersburg, Md.). After washing, the blot was subsequently probed with an alkaline phosphatase conjugated antirabbit IgG (BioRad, Hercules, Calif.) at a 1:7000 dilution in TBST with 5% skim milk for 1 h. Finally, antibody binding was detected with CSPD according to the manufacturer's instructions (BMB, Indianapolis, Ind.) and the blot was exposed to X-ray film (Hyperfilm, Amersham).

Potato tuber moth bioassay

Potato tuber moth rearing and detached leaf bioassays were performed according to Douches et al. (1998). The *cryV-Bt*/*PVY°cp*-transgenic plants, nontransgenic cultivars, a *cryV-Bt* transgenic 'Spunta' (G3), and a *PVY°cp*-transgenic 'Atlantic' (Y1) were transplanted to a greenhouse with a 18 to 25 °C temperature range and a 16-h photoperiod (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$). When plants were at a six-leaf stage, 10 first instar larvae were placed on young detached leaves near the midrib in a 20 × 150 mm petri dish. The petioles of the excised leaves were placed in water-filled vials that were sealed with a sponge to maintain plant turgor. The petri dishes were covered and placed in a 25 ± 2 °C room with 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light. Each dish represented one replication and each line was replicated four times. Mortality of the potato tuber moth larvae was determined after 72 h. Most mortality occurred in the first instar (1 mm), and when larvae had died and desiccated, it was not always possible to find the remains; therefore, missing larvae were considered dead.

PVY° greenhouse bioassay

The *cryV-Bt*/*PVY°cp*-transgenic plants, nontransgenic cultivars, and a PVY°cp-transgenic 'Spunta' were transplanted to a greenhouse with a 18 to 25 °C temperature range and a 16-h photoperiod (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$). When plants were at the five leaf stage, two well-expanded, topmost leaves were dusted with carborundum (400 mesh) and rubbed with 150 µL sap (extracted using a mechanical grinder) from 2 g of potato leaves infected with PVY° virus and diluted 10:1 v/v with distilled water. After rubbing, the inoculated leaves were immediately rinsed with water. As a high selection stress, virus replication and spread were enhanced in these plants by maintaining a higher temperature in the greenhouse (25 to 27 °C). Leaves were collected and analyzed by a double antibody sandwich enzyme-linked immunosorbant assay (DAS ELISA) using an anti-PVY antibody from Agdia Inc. (Elkhorn, Ind.), according to manufacturer's instructions, for the extent of PVY virus infection 30 and 45 d postinoculation (DPI). Absorbance values were read on a Bio-Tek Instrument EL-307 at 405 nm. The transgenic lines were defined as susceptible to PVY if the ratio of the optical density (O.D.) absorbance (405 nm) mean of the transgenic line to the O.D. mean of healthy control was 4:1 or greater (McDonald et al., 1994).

Results and Discussion

Transformation and identification of transgenic plants

To introduce resistance to potato tuber moth and PVY, the *cryV-Bt* and *PVY°cp* genes were transformed into potato, an economically important cultivar grown in North Africa. Shoots emerged from callus after 30 d culture on regeneration medium supplemented with 50 $mg \cdot L^{-1}$ kanamycin and 200 $mg \cdot L^{-1}$ Timentin. More than thirty kanamycin-resistant plants were obtained from each construct. The putative integration of the *cryV-Bt* and *PVY°cp* genes in 'Spunta' lines was assayed initially by PCR (Fig. 2). Southern analysis was carried out on five PCR-positive *cryV-Bt*/*PVY°cp*-transgenic lines (with pBIML6a T-DNA), one PCR-positive PVY-transgenic line (with pBI-PVY T-DNA), and one *cryV-Bt* transgenic line (with pBIML5 T-DNA) (Fig. 3). Southern analysis also determined that each *cryV-Bt*/*PVY°cp*-transgenic line tested contained at least one or two copies of the *PVY°cp* gene per tetraploid genome (Fig. 4). To determine copy number a PVY probe was hybridized to genomic DNA digested with *BamHI*, which digests upstream of the PVY gene.

Expression of *cryV-Bt* and *PVYcp* genes in transgenic potato plants

Transgenic plants containing pBIML6a (*cryV-Bt*/*PVY°cp*), pBI-PVY (*PVY°cp*), and pBIML5 (*cryV-Bt*) were analyzed on northern blots for *cryV-Bt* and *PVY°cp* transcription. The expected size for the *cryV-Bt* and *PVY°cp* mRNAs are 2.2 and 1.2 kb,

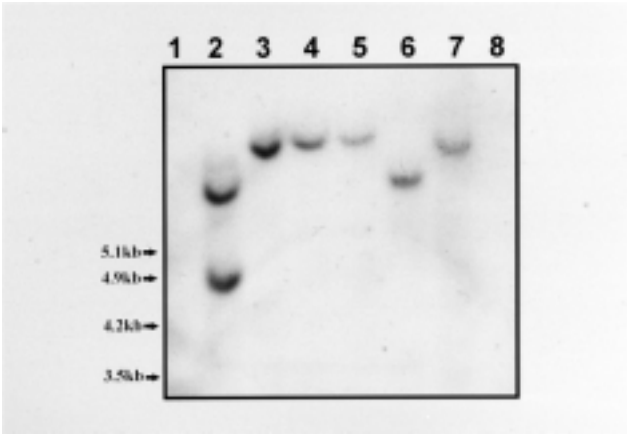

Fig. 4. Southern analysis of gene copy number. DNA digested with *Bam*HI and hybridization with the *PVY°cp* gene. Lane 1 = Spunta (nontransgenic plant); lanes 2 to 6 = 6a-1 to 6a-55; lane 7 = Y-1 (transgenic plant with *PVY°cp* gene only), lane 8 = G-3 (transgenic plant with *cryV-Bt* only).

respectively. The *cryV-Bt* and *PVY°cp* transcription levels in the transgenic potato plants, isolated from both leaf and tuber organs, are shown in Fig. 5 and Fig. 6, respectively. Both *cryV-Bt* and *PVY°cp* mRNA accumulated in the leaves, but the levels of transcript in the tuber of *cryV-Bt* in the *cryV-Bt/PVY°cp* transgenic lines were less than the *cryV-Bt* (no *PVY°cp* gene) line (G3). Moreover, the *PVY°cp* transcript in the tuber was more consistent and greater among the lines than the *cryV-Bt* transcripts across the *cryV-Bt/PVY°cp* transgenic lines. One possibility for this observation is that some tuber-specific genes may interfere with the expression of the *cryV-Bt* gene in certain positions in the genome. Of these lines, 6a-1, which had two copies of the double gene construct, had a much higher *PVY°cp* transcript level and a much lower *cryV-Bt* level. As expected in the transgenic lines with the pBIML5 construct (*cryV-Bt*), only mRNA for *cryV-Bt* was detected and in the transgenic lines using the *PVY°cp* construct, only *PVY°cp* mRNA was detected.

Western blot analysis was used to determine the *cryV-Bt* protein accumulation in transgenic plants. A total of five *cryV-Bt/PVY°cp*-transgenic lines and two *cryV-Bt*-transgenic lines (G2 and G3) contained detectable amounts of the 81.2kDa (Sekar et al., 1997) *cryV-Bt* protein (Fig. 7). A band of lower molecular weight also reacts with the *cryV-Bt* antibody. This lower band appears to be nonspecific binding because it is present in the nontransgenic controls. The matching Coomasie stained gel (data not shown) revealed that equal amounts of protein were loaded for all samples except for G2 (lane 8), which had 25% more protein. The level of *cryV-Bt* protein expression in the *cryV-Bt/PVY°cp*-transgenic lines varied. For example, the *cryV-Bt*-transgenic (G3) line contained significantly more *cryV-Bt* protein than the other *cryV-Bt*-transgenic (G2) as well as the five *cryV-Bt/PVY°cp*-transgenic lines. Line 6a-1 exhibited a lower protein level than the other four *cryV-Bt/PVY°cp*-transgenic lines. Northern analysis (Fig. 5) showed that line 6a-1 had less transcript levels than the other lines. One explanation for the low *cryV-Bt* transcript and protein levels is that the position of its transgene in the genome could be effecting it trancriptionally and translationally.

Insecticidal activity of *cryV-Bt* gene in transgenic plants

Seven transgenic lines (5 *cryV-Bt/PVY°cp*, 1 *cryV-Bt* and 1 *PVY°cp*) and 'Spunta' were screened for insecticidal activity using a leaf bioassay against neonate potato tuber moth larvae. All *cryV-Bt* transgenic lines (alone or in combination with *PVY°cp* gene) were lethal to potato tuber moth larvae with 70% to 87% mortality (Table 1). Conversely, in the *PVY°cp*-transgenic line and nontransgenic 'Spunta', mortality was 10% to 13%. In general, the larvae that fed on transgenic plants containing the *cryV-Bt/PVY°cp* or *cryV-Bt* construct were severely restricted in growth, development, and leaf mining with up to 87% mortality during the first three days of feeding. This level of potato tuber moth mortality was similar to or higher than the levels reported by Douches et al. (1998) using the *cryV-Bt*/GUS fusion gene alone. Other reports showed that the highest mortality for PTM is >70% by using the *cryIA*(b) or *cryIA*(b)6 (Van Rie J. et al., 1994). We also observed a range of potato tuber moth mortality among the *cryV-Bt/PVY°cp*-transgenic lines (70% to 87%). Even though 100% mortality was not achieved in the 72-h assay, larval growth was restricted and all larvae were dead after five days of feeding (data not shown). Transgenic line 6a-1, which expressed the lowest level of *cryV-Bt* protein in the western analysis, also exhibited significantly lower mortality of potato tuber moth (Fig. 7, Table 1), suggesting that the concentration of toxic protein in transgenic tissue is, as expected, strongly correlated with insect lethality. *CryV-Bt* and *PVY°cp* genes, in our research, were both independently regulated by the CaMV 35S promoter. Since the same promoter was used, homology exists between the two promoters and could therefore lead to *cryV-Bt* gene transcriptional silencing in some transgenic lines (Matzke and Matzke, 1995). This could be one reason for the range of PTM mortality among *cryV-Bt/PVYcp* transgenic plants (70% to 87%).

Susceptibility of transgenic potato plants to infection by PVY°

To evaluate PVY virus resistance, *cryV-Bt/PVY°cp*-transgenic lines and *PVY°cp*-transgenic lines (Y-1, Atl-5) were challenged by sap inoculation of the PVY° strain and compared to nontransgenic 'Spunta' and 'Atlantic'. To determine whether the PVY antibody would react with the transgenic coat protein, Spunta *cryV-Bt/PVY°cp*-transgenic lines, which were not infected with the virus were compared to the nontransgenic 'Spunta' and 'Atlantic'. The uninfected transgenic *cryV-Bt/PVY°cp*-transgenic lines gave O.D. absorbance values equal to the uninfected nontransgenic lines (data not shown). As shown in Table 2, all transgenic lines tested, except line 6a-4, manifested moderate to high tolerance to PVY virus infection at 30 DPI, while the control plants were infected with PVY. The symptoms paralleled PVY titer levels (data not shown). A range of PVY tolerance was observed among the *cryV-Bt/PVY°cp*-transgenic lines. Of the five

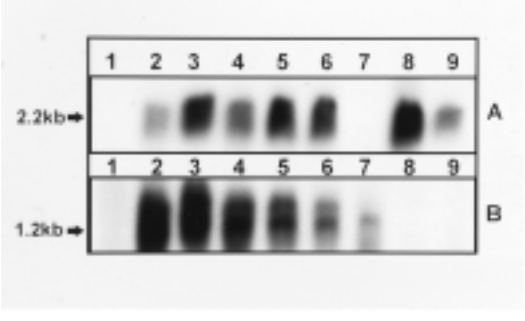

Fig. 5. Northern analysis for leaf tissue. (**A**) Total leaf RNA hybridized with *cryV-Bt* gene RNA probe. (**B**) Total RNA hybridized with *PVY°cp* gene RNA probe. Lane 1 = Spunta (nontransgenic plant); lanes 2 to 6 = 6a-1 to 6a-5; lane 7 = Y-1 (transgenic plant with *PVY°cp* gene only); lanes 8 and 9 = G-2 and G-3 (transgenic plants with *cryV-Bt* only).

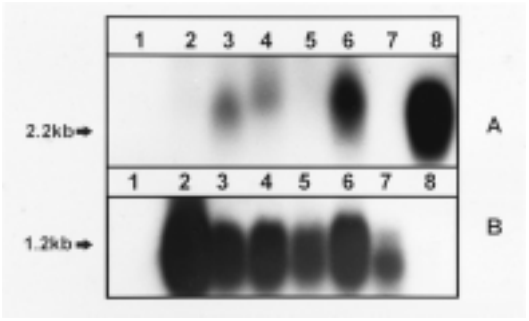

Fig. 6. Northern analysis for tuber tissue. (**A**) Total tuber RNA hybridized with *cryV-Bt* gene RNA probe. (**B**) Total RNA hybridized with *PVY*^{o}*cp* gene RNA probe. Lane 1 = Spunta (nontransgenic plant); lanes 2 to 6 = 6a-1 to 6a-5; lane 7 = Y-1 (transgenic plant with *PVY*^{o}*cp* gene only); lane 8 = G-3 (transgenic plant with *cryV-Bt* only).

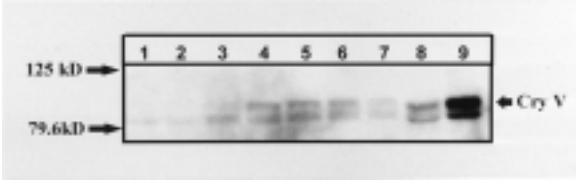

Fig 7. Western immunoblot analysis of transgenic and nontransgenic plants using *cryV* antibody. Lanes 1 and 2 = Spunta, USDA8380-1 (nontransgenic plants); lanes 3 to 7, 6a-1 to 6a-5; lanes 8 and 9 = G-2 and G-3 (transgenic plants with *cryV-Bt* only).

cryV-Bt/*PVY*^{o}*cp*-transgenic lines, 6a-3 and 6a-5 expressed the highest tolerance to PVY 30 DPI. After 45 d postinfection, these two transgenic lines still had low PVY titer levels. This level of response was as effective as the PVY tolerance observed with the *PVY*^{o}*cp*-transgenic lines (Y-1 and Atl-5).

Lawson et al. (1990) achieved complete resistance to PVX and PVY in potato using viral coat protein genes. In comparison, our control of PVY was relatively incomplete. PVY titer levels increased as DPI increased (Table 2). This could be due to the *PVY*^{o}*cp* gene itself. The *PVY*^{o}*cp* gene we used in our research was different from those used by Lawson et al. (1990) and Smith et al. (1995). Our *PVY*^{o}*cp* lines (Table 2) as well as other *PVY*^{o}*cp* lines (unpublished observation) did not show a greater resistance to PVY infection compared to our best *cryV-Bt/ PVY*^{o}*cp* line. It may be that this *PVY*^{o}*cp* gene is not as effective as the other previously cloned *PVY*^{o}*cp* genes on cp translation level or on the extent of posttranscription gene silencing.

Initially, it was thought that it was the coat protein that conferred resistance to potato virus Y (Lawson et al., 1990). It was later shown by Smith et al. (1995) that untranslatable PVY coat protein constructs produced resistant lines as well. They found that the *PVYcp* transformants, which were highly resistant, had multiple copies of the transgene and a low steady state level of transcripts. Multiple copies of the *PVY*^{o}*cp* gene may also appear to be attractive targets of methylation, which could be related to the steady state of the transgene (Smith et al., 1994). According to our Southern analysis (Fig. 4), only one *cryV-Bt/ PVY*^{o}*cp* transgenic line, 6a-1 had more than one copy (located in different positions) of the *PVY*^{o}*cp* gene. Smith et al. (1995) also showed that there is evidence for an RNA-mediated response for resistance. Sijen et al. (1996) have shown that RNA-mediated resistance is associated with posttranscriptional gene silencing which involves very specific degration of both the transgene mRNA and the similar or complementary viral RNA. We observed a strong visual signal for RNA transcripts in *cryV-Bt/ PVY*^{o}*cp* transgenic lines (Fig. 5), but the variation in resistance to PVY is significant among these transgenic plants according to our ELISA results (Table 2). Additionally, we observed moderate to high resistance levels with high transcription levels, however it was not a complete resistance. It is therefore likely that the coat protein plays a moderate role in the resistance levels in our study. The low number of copies, copies not closely linked and possibly poor coding sequence homology between our *PVY*^{o}*cp* lines and the PVY viral RNA could account for our variable and incomplete resistance to PVY^{o} infection.

The results described above demonstrate that the cotransformation of *cryV-Bt* and *PVY*^{o}*cp* genes in an economically important potato cultivar is effective against both potato tuber moth and PVY. We identified two *cryV-Bt*/*PVY*^{o}*cp* transgenic lines, 6a-3 and 6a-5, which possess high tolerance to PVY infection as well as a strong resistance to potato tuber moth. The insect bioassay and virus inoculation assays were laboratory and greenhouse based, respectively. Beneficial factors present in the field such as predators and adverse environmental conditions for the insect and virus will contribute to their control but were not factors considered in the current lab and greenhouse experiments. Thus, a stringent field test of these transgenic plants with potato tuber moth and PVY infestation will be conducted during the upcoming years in subtropical regions, where tuber moth and PVY are major pest problems (i.e., Egypt), to fully test their efficacy.

Table 1. Feeding assay of transgenic lines with potato tuber moth.

Construct	Transgenic lines	Mortality[z] (%)	Mining[y]
pBIML6a	6a-1	70 b	N
	6a-2	77 c	N
	6a-3	87 d	N
	6a-4	80 c	N
	6a-5	80 c	N
pBIML5	G-3	83 cd	N
pBI-PVY	Y-1	10 a	Y
Spunta[x]		13 a	Y

[z]Mortality was tested by Fisher's protected LSD. Mortality means with the same letter are not significantly different ($P < 0.05$).
[y]N = mining ceased during the 72-h feeding period; Y = mining continued for the duration of the 72-h feeding period.
[x]Nontransgenic control.

Table 2. PVY^{o} suspect analysis of transgenic lines by DAS ELISA.

Transgenic lines	PVY^{o} suspect of DAS ELISA[z] (d) 30	45
6a-1	++[z]	+++
6a-2	++	+++
6a-3	+	++
6a-4	+++	+++
6a-5	+	++
Y-1	+	++
Atl-5	+	++
Spunta[y]	+++	+++
Atlantic[y]	+++	+++

[z]+++ = susceptible (titer in inoculated plant : healthy plant >4:1); ++ = tolerant (titer in inoculated plant:healthy plant 3 to 4:1); + = highly tolerant (titer in inoculated plant:healthy plant 12:1).
[y]Nontransgenic plants.

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