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Expression of *Bacillus thuringiensis* (B.t.) insecticidal crystal protein gene in transgenic potato

Ming-Tsair Chan[1,4], Liang-Jwu Chen[2], and Hsin-Hsiung Chang[3]

[1]*Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan 11529, Republic of China*

[2]*Institute of Molecular Biology, National Chung Hsing University, Taichung, Taiwan, Republic of China*

[3]*Department of Agronomy, National Taiwan University, Taipei, Taiwan 10764, Republic of China*

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Abstract. The crystal proteins, δ-endotoxins, of *Bacillus thuringiensis* are specifically lethal to Lepidopteran insects. A truncated B.t. toxin gene, *cry*IA(a), encoding an insecticidal crystal protein (ICP) directed by the cauliflower mosaic virus 35S promoter was transferred to potato plants by an *Agrobacterium*-mediated transformation system. The integration of the *cry*IA(a) gene into potato genome was determined by Southern blot analysis and polymerase chain reaction (PCR). The copy number of the integrated gene was estimated by inverse polymerase chain reaction (IPCR). The *cry*IA(a) RNA transcripts in transgenic potato plants were demonstrated by Northern blot analysis. Seven out of thirty transgenic plants expressed the *cry*IA(a) gene. Those transgenic plants containing multiple transgene copies did not express *cry*IA(a) gene. Nevertheless, transgenic potato plants grown in the greenhouse contained 7–52 ng ICP per gram fresh leaf.

Keywords: *Agrobacterium tumefaciens*; *Bacillus thuringiensis*; *cry*IA(a) gene expression; Insecticidal crystal protein; Transgenic potato.

Abbreviations: ICP, insecticidal crystal protein; **IPCR**, inverse polymerase chain reaction; **PCR**, polymerase chain reaction; **PSC**, potato suspension cultures; **RB**, T-DNA right border; **LB,** T-DNA left border; **NPTII**, neomycin phosphotransferase II.

Introduction

The potato (*Solanum tuberosum* L.) is one of the major crops in agricultural production. Current efforts to develop insect-resistant crops through biotechnology are based primarily on transforming plants with a single gene encoding insecticidal enzyme or toxin. The most widely used genes in this approach are the δ-endotoxin gene of *Bacillus thuringiensis*, a sporeforming, gram-positive bacterium. The insecticidal crystal protein (ICP) from the *B. thuringiensis* var. *kustaki* is a specific toxin for lepidopteran insects yet exhibits no toxicity toward humans, other vertebrates, or beneficial insects (Delannay et al., 1989). Formulated bacterial products have been used as insecticides for a long time. However, practical usages of such microbial products are limited because of their relatively high cost and poor persistence under field conditions, resulting in a need for multiple applications (Sneh et al., 1983).

Lepidopteran-active ICPs are protoxins of MW. 130–160 kDa. These protoxins emerge when exposed to an alkaline medium (pH 9–12), such as that found in the insect midgut. These protoxins are proteolytically cleaved into smaller, active forms (MW 60–70 kDa) derived from the N-terminal half of the protein (Hofte et al., 1989). Although the mode of toxin action is largely unknown, it is assumed to bind specific proteins on the membrane of the insect gut (Hofmann et al., 1988).

The B.t. toxin *cry*IA gene of *B. thuringiensis* has been engineered and transferred into several plant species to yield resistance against certain lepidopteran insects. The truncated genes, which produce insecticidally active protein, have been expressed in potato (Adang et al., 1993; Perlak et al., 1993), tomato (Delannay et al., 1989), tobacco (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987), cotton (Perlak et al., 1990), corn (Koziel et al., 1993), and rice (Fujimoto et al., 1993). The use of a native δ-endotoxin coding region, which has a high A-T content, appears to lead to an abnormally low expression in plants. Modifications of the coding region sequence to increase the G-C content of the native gene resulted in a dramatic increase in the expression of the insecticidal protein (Perlak et al., 1991).

As a first step toward the development of an insect resistant potato, attempts were made to transfer the truncated *cry*IA(a) gene directed by the cauliflower mosaic virus 35S promoter into potato plants through *Agrobacterium*-mediated transformation. These transgenic potato plants could provide alternatives to hazardous synthetic chemical insecticides for controlling lepidopteran pests.

[4]Corresponding author.

Materials and Methods

Plasmid Construction

The 2 kb truncated *cry*IA(a) gene was isolated from the plasmid DNA of *B. thuringiensis* var. *karstaki* by PCR (Schnepf et al., 1985). Two sequences in the B.t. coding region were chosen to amplify a 2 kb fragment within the gene. These two sequences were: 5' primer (TGGAGGTAACTTATGGATAACAATCCG) and the 3' primer (TCACTCAACTAAATTGGATACTTGATCA). The 5' and 3' primers include a plant translation initiation site (ATG) and a stop codon (UGA), respectively. PCR was carried out in a 50-ml reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.25 mM $MgCl_2$, 0.01% (W/V) gelatin, 0.1% (W/V) Triton X100, 0.2 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), and 2.5 units of *Taq* DNA polymerase (Promega). The sample was preheated at 94°C for 1.5 min; annealed at 37°C for 2 min; and extended at 72°C for 5 min. This process was followed by 13 cycles of denaturation at 94 °C for 1 min; annealing at 40°C for 2 min; and extension at 72°C for 5 min. The PCR amplified 2 kb DNA fragment containing the essential region, the N-terminal region, of the B.t. gene was then modified to blunt end with Klenow fragment before ligation with pTZ19U plasmid at the *Sma*I site. The resulting clone U73 was then used as a source of the B.t. gene. The B.t. gene *Hinc*II-*Sst*I (*Sst*I partial digestion) restriction fragment from the U73 was subcloned into the *Sma*I and *Sst*I site of the plant expression vector pBI121 (Jefferson, 1987) in which the GUS gene was deleted to create the new plasmid pBT121A (Figure 1). The pBT121A plasmid contained NPTII coding sequence for transformant selection in a kanamycin medium.

Transgenic Potato Plants

Triparental mating was used to mobilize pBT121A constructs into *Agrobacterium tumefaciens* C58C1 harboring helper plasmid pGV2260 (which provides *vir* functions) (kindly provided by Dr. Marc Van Montagu, Laboratorium voor Genetica, Belgium). Potato (*Solanum tuberosum* L.) cv. ADH69 microtubers grown *in vitro* were transformed with *Agrobacteria*, and kanamycin-resistant plants were regenerated (Chang and Chan, 1991).

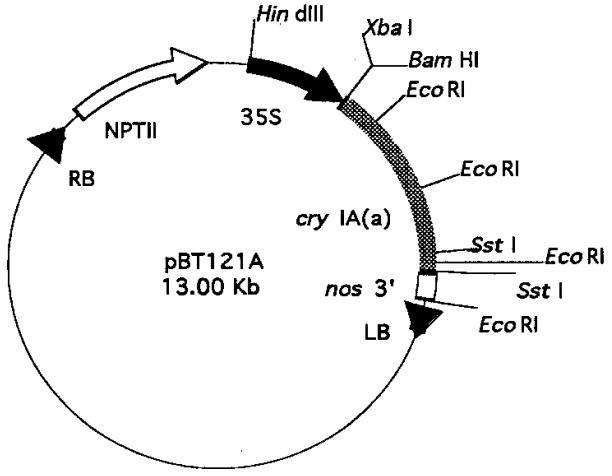

Figure 1. Map of the binary vector pBT121A containing the 35S/*cry*IA(a) chimeric gene.

DNA Analysis

DNA was isolated from leaves of putative transgenic plants according to the CTAB method (Hurray and Thompson, 1980). DNA blot analysis was performed as described by Maniatis et al. (1982). About 0.8 kb of the *cry*IA(a) DNA was used as probe generated from the *Eco*RI digestion of pBT121A and labeled with [α-^{32}P]dCTP using the random primer method (Feinberg and Vogelstein, 1983). To determine the copy number of transgenes integrated into transgenic plants, inverse polymerase chain reaction (IPCR) of genomic DNA for NPTII DNA was performed according to the method previously described (Does et al., 1991).

cryIA(a) RNA Analysis

Total RNA was purified from leaves according to the method of Belanger et al. (1986). RNA blot analysis was performed as described by Thomas (1983). The *Eco*RI fragment of pBT121A containing the *cry*IA(a) DNA was used as a probe.

NPTII Dot Blot Assay

The NPTII activity in the putative transgenic plants was assayed for at least three replicates using the method described by Chan et al. (1993).

ICP Protein Determination

Truncated ICP was used as a standard on the immunoblot. This protein was produced under the influence of the lac promoter on pUN4 (Chen, 1992). Polyclonal rabbit antibodies specific for the *B. thuringiensis* subsp. *kurstaki* ICP were used to determine the quantity of ICP accumulated in the crude extract of leaf samples. Extracts were made by grinding leaf tissue in liquid nitrogen followed by addition of extraction buffer (50 mM Na_2CO_3, pH 9.5, 100 mM NaCl, 0.05% Triton X-100, 0.05% Tween-20, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 mM leupeptin). Protein contents were determined using the Bradford method (Bradford, 1976). Western blot analysis was done as described by Yu et al. (1991). Protein dot-blot analysis was performed as described previously (Chan et al., 1994).

Results

Introduction of a Truncated cryIA(a) Gene into Potato Plants

Three to four month old microtubers grown in vitro were inoculated with *Agrobacterium tumefaciens* C58C1 (pGV2260 + pBT121A) and then cultured on a kanamy-

cin selection medium. Thirty putative transformants out of the one-hundred treated microtubers were obtained and designated as P1 to P30. Southern blot analysis of *Hin*dIII digested genomic DNA from leaves of three putative transformants (P5, P7, and P10) were performed in order to demonstrate the integration of 35S/*cry*IA(a) in the genome of ADH69 (Figure 2). One band of approximately 11 kb appeared for P5 (Figure 2A, lane 2), 14 kb for P7 (Figure 2A, lane 3), and 6.5 kb for P10 (Figure 2A, lane 4). The probe did not hybridize with the DNA of the non-transformed control (Figure 2A, lane 5). These results indicate that the *cry*IA(a) DNA was integrated into the genome of the transformants. All other putative transformants were evaluated to determine whether the chimeric gene was integrated into the genome and to determine its copy number by IPCR analysis. IPCR analysis of genomic DNA using primers for amplifying the NPTII gene showed that most of the transformants—except P13, P16, P22, and P23— contain one copy of NPTII genes. No amplified DNA fragment was obtained for the non-transformant or for one putative transformant, P4 (data not shown). It is possible that P4 might be a non-transformant escaped from the selection medium. P13 and P23 showed two copies of the chimeric gene (Figure 2B) while P16 and P22 showed three and four copies, respectively (Figure 2B). When using DNA samples of the remaining 29 transgenic potatoes as template and priming them with the 3' and 5' ends of the *cry*IA(a) gene for PCR analysis, only 24 transformants had the amplified fragment (data not shown).

Expression of 35S/cryIA(a)/nos in Potato Variety ADH69

To examine the expression of the *cry*IA(a) gene in the 24 transgenic potato plants, total RNA isolated from leaves was hybridized with the coding region of the truncated *cry*IA(a) gene. The results indicated that *cry*IA(a) RNA transcripts were present in leaves of seven transgenic potato plants and the level of expression varied (Figure 3). No RNA transcript could be detected in P16. The remaining 17 transformants, including P13, P22, and P23, did not hybridize with the probe (data not shown). The NPTII transcript could be detected when these blots were rehybridized with a NPTII probe, indicating that the total RNA was not degraded (data not shown). In addition, staining of ribosomal RNA (rRNA) with ethidium bromide showed that the amount of total RNA applied was approximately the same among the different transgenic plants (Figure 3). The results of Northern blot analysis imply that *cry*IA(a) RNA can not be expressed well in those transgenic plants containing multiple transgene copies (P13, P16, P22, and P23); however, the NPTII RNA transcript can be detected in these transformants.

Expression of NPTII in the Transgenic Potato Plants

Plasmid pBT121A containing the NPTII-coding region was driven by the nopaline synthase promoter. Accordingly, selection for plants carrying the foreign genes was achieved using media containing kanamycin. To determine if the NPTII mRNA resulted in the synthesis of

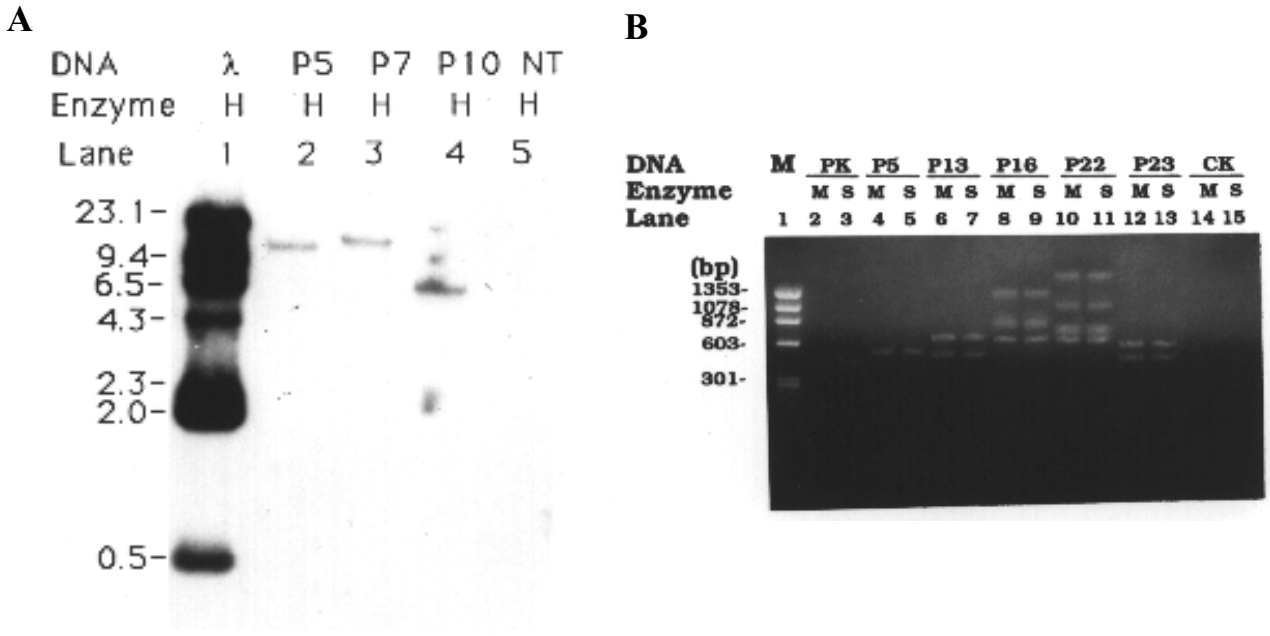

Figure 2. (A) DNA blot analysis for detection of *cry*IA(a) DNA in the putative transgenic potato plants. Five μg of DNA digested with *Hin*dIII were loaded into each well. The *Eco*RI 0.8 kb fragment from pBT121A containing *cry*IA(a) DNA was used as a probe. Lane 1, λDNA cut with *Hin*dIII; Lanes 2–4, independent transgenic plant, P5, P7, P10, respectively. Lane 5, DNA from a non-transformed control plant. (B) Analysis of the IPCRs for 5 transformed plants (P5, P13, P16, P22, P23) and a non-transformed control plant (CK). One μg of plant DNA was used per reaction. Lane 1, ϕX174 marker; Lanes 2–3, plasmid pBT121A as the positive control (PK). M: *Mst*II; S: *Sst*II.

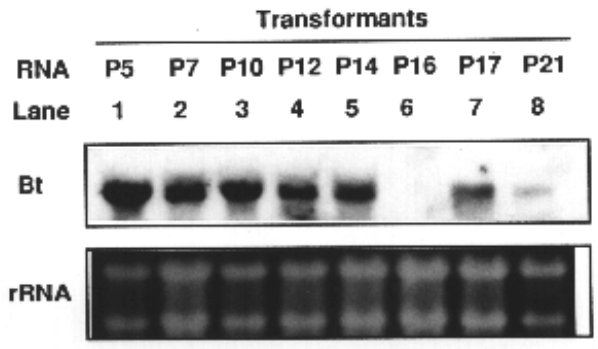

Figure 3. Northern blot of total RNA from transgenic potato leaves hybridized with the *cry*IA(a) DNA fragment. Ethidium bromide staining of gel prior to blotting showed that RNA was intact as judged by ribosomal RNA (rRNA) bands and that each lane contained an approximately equal amount of RNA. B.t. = *cry*IA(a).

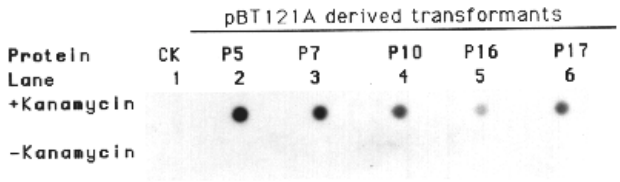

Figure 4. Neomycin phosphotransferase II dot blot assay. Thirty μg protein extracts from leaves and extracts of 5 randomly selected transgenic potato plants were reacted with [γ-^{32}P]-ATP, dot blotted on Whatmann P81 papers and autoradiographed. Row A: reactions with kanamycin. Row B: reaction without kanamycin. Lane 1, protein extracts from non-transformed control plant (CK); Lanes 2–5, protein extracts from transgenic potato, respectively.

NPTII protein, protein was extracted from leaves of transgenic plants. NPTII activity was further monitored in 15 randomly chosen transgenic plants. All of the 15 transgenic plants demonstrated NPTII activity. No activity was observed in the non-transformed control group (Figure 4). These results clearly demonstrated that NPTII protein can be transcribed well in these transgenic plants.

Expression of CryIA(a) Protein in Transgenic Potato Plants

To determine the expression of CryIA(a) protein, immunoblot analysis was performed with extracts obtained from leaves of transgenic potato plants to ascertain levels of the CryIA(a) protein accumulated in transgenic plants. No signal, except the purified CryIA(a) protein from *E. coli* (the positive control), was detected for all transformants. The lack of signal could be attributable to a low level expression of the *cry*IA(a) gene in transgenic plants. Therefore, a high protein concentration (500 μg) was applied to the dot-blot apparatus and subjected to immunoblot analysis. In addition, we chose six transgenic plants in which *cry*IA(a) mRNA expression could be achieved and P16, no *cry*IA(a) mRNA transcript, to be the materials As shown in Figure 5, the CryIA(a) protein was detectable in six transformants under this condition. P10 transformant produced the highest levels of CryIA(a) protein compared to the others listed in the Table 1. The yield of the CryIA(a) protein was estimated to be about 52 ng per g of fresh leaf tissue in P10, which was about six times higher than plant P17. P16, which contained the *cry*IA(a) gene but had no protein according to immunoblot analysis, produced no detectable level of ICP in the plant (Table 1). No cross reaction could be observed with a non-transformed control plant.

Table 1. Comparison of *cry*IA(a) ICP levels in independent transgenic potatoes.

ICP line	ng ICP/g fresh weight (mean ± SEM)
P5	41 ± 5
P7	39 ± 6
P10	52 ± 7
P12	32 ± 8
P14	28 ± 7
P16	nd
P17	7 ± 4
P21	11 ± 3
CK	2 ± 1

*Values were obtained by dot-blot and densitometry assay and converted to ng ICP per gram fresh weight. Leaf samples were obtained from nodes 1 to 5. Values shown are average of 4 samples. nd: not detectable; CK: non-transformant.

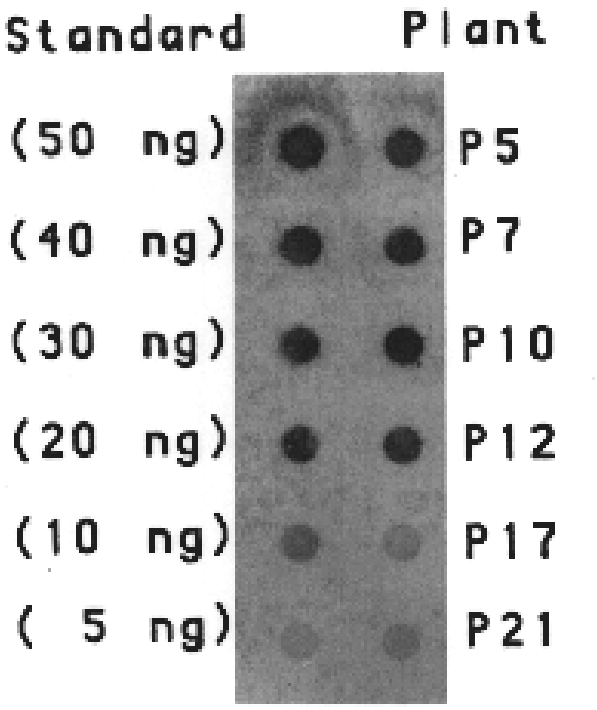

Figure 5. ICP protein dot blot assay. Protein extracts (500 μg) from approximately one gram of transgenic potato leaf tissue were dot blotted on nitrocellulose membrane, and the ICP protein was detected by an alkaline phosphatase conjugated goat anti-rabbit antibody after the binding of an antibody against ICP.

Discussion

The 35S/*cry*IA(a)/nos chimeric gene was transferred into and expressed in potato plants. Twenty-nine transformants which survived on selection medium, expressed the NPTII activity (Figure 4). Using DNA samples of the 29 transgenic plants as templates to amplify the *cry*IA(a) gene fragment, five transformants showed no evidence of amplification. Although the possible cause of this phenomenon is still unclear, it is possible that the gene might be lost due to the replication and repair of transgenes prior to integration (Gheysen et al., 1991).

Most transgenic plants carrying the *cry*IA(a) gene did not express it with the exception of seven transformants (Table 1). The correlation between gene copy number and its expression in transformants has been reported to be positive (Gendloff et al., 1990; Hobbs et al., 1992), indeterminate (Dean et al., 1989), or negative (Hobbs et al., 1990, 1992). The results in this study indicated that transformants with higher ICP activity all had single copy while those transformants with no ICP activity (like P13, P16, P22, and P23) all had multiple copies of the T-DNA containing *cry*IA(a) gene (Figure 2B). We do not know what causes this phenomenon. In this experiment the rRNA was used as an internal control and a similar amount of RNA was loaded into each well. This negative correlation suggested a possibility that the lack of *cry*IA(a) mRNA transcripts might result from epigenetic silencing of gene expression by induction of repeated DNA sequence. A similar observation was reported earlier by van der Krol et al. (1990) and Napoli et al. (1990), who showed that transformation of additional homologous genes caused a gene-specific collapse in expression. The mechanism of co-suppression by transgenes may involve interference of RNA strands with the transcription process itself or DNA methylation of the endogenous gene. DNA methylation has been shown to be a mechanism for inactivation of chimeric transgenes (Hobbs et al., 1990; Matzke et al., 1989) and the demethylating agent 5-azacytidine has been used to reactivate silent transgenes (Bochardt et al., 1992). In addition, treatment of *Agrobacterium* with 5-azacytidine was efficient in increasing transformation frequencies (Palmgren et al., 1993). The suppression of *cry*IA(a) caused by these processes is being studied in our laboratory.

Among the seven transformants which expressed *cry*IA(a) gene, the levels of mRNA transcripts varied. The variation in transgene expression might be the result of a position effect in the genome. Low levels of *cry*IA(a) gene expression in plants might also be attributable to mRNA instability as suggested by Murray et al. (1991). A similar construct, 35S/*cry*IA(a)/nos, was involved in their studies. This RNA instability might also be due to an incomplete functioning of a polyadenylation signal. Furthermore, plant codon usage, in general, prefer G + C content in the codon position III (Murray et al., 1989), but the truncated *cry*IA(a) gene used in our study has a high A + T content, which may lead to low gene expression. However, a major block to *cry*IA(a) gene expression in plants might be related to translation, which in turn effects the accumulation of *cry*IA(a) mRNA. Several lines of evidence show that insect-resistant plants containing the modified *cry*IA(b) have higher amounts of RNA than those with the truncated wild type gene (Fujimoto et al., 1993; Perlak et al., 1991). Those modified ICPs are more abundant in the transgenic plants.

Transgenic plants expressing insecticidal crystal genes are a powerful tool in an integrated pest management program. Several strategies have been proposed to increase insect-resistance in the field. The first involves the use of a tissue-specific, chemically-responsive (Williams et al., 1992), or wound-inducible promoter for B.t. expression. The second strategy is to modify the B.t. coding usage leading to a higher expression in the plant. The third, and final, strategy for enhancing the insect-resistant effect is to induce expressions of various B.t. genes in the same plant. Although in our study the level of CryIA(a) in transgenic potato plants was relatively low, it still amounted to 7–52 ng/g of fresh weight tissue. Several studies have also shown other transgenic plants expressing the truncated CryIA(b) at a level similar to our transgenic potatoes (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987). Since the LC_{50} (50% insect lethal of ICP concentration) for lepidopteran pests is about 25–40 ng/g (Vaeck et al., 1987), it is highly possible that transgenic potato plants will have significant defences against lepidopteran pests with further improvement in gene expression.

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蘇力菌殺蟲晶體蛋白基因在轉殖馬鈴薯的表現

詹明才[1] 陳良築[2] 張新雄[3]

[1]中央研究院分子生物研究所

[2]國立中興大學分子生物研究所

[3]國立臺灣大學農藝學系

蘇力菌殺蟲晶體蛋白一內毒素，是一種對鱗翅目昆蟲有專一致死性的蛋白質。我們將縮短的蘇力菌殺蟲晶體蛋白基因 *cry*IA(a)，放在花椰菜鑲嵌病毒 35S 啟動子之後，以農桿菌轉殖系統轉殖進入馬鈴薯。*cry*IA(a) 基因嵌入馬鈴薯染色體組中，已由南方墨點法及聚合酶鏈鎖反應證實，外來基因的嵌入數目則由反轉聚合酶鏈鎖反應 (IPCR) 決定。轉殖馬鈴薯中 *cry*IA(a) RNA 轉錄情形由北方墨點法檢測。三十株中只有七株轉殖馬鈴薯表現 *cry*IA(a) 基因，基因的表現量也很低。帶有複基因的轉殖植物都不表現 *cry*IA(a) 基因。然而，生長在溫室中的轉殖馬鈴薯每克葉片鮮重中約有 7-52 ng 殺蟲晶體蛋白的表現量。這些結果證實修飾性的 *cry*IA(a) 基因對於發展抗蟲馬鈴薯品種會是一種有用的基因改造工具。

關鍵詞：蘇力菌；蘇力菌殺蟲晶體蛋白；轉殖馬鈴薯；農桿菌；*cry*IA(a) 基因表現。