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Expression of a *Bacillus thuringiensis cry1C* gene in plastid confers high insecticidal efficacy against tobacco cutworm - a *Spodoptera* insect

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Abstract. We have demonstrated that the toxic domain of a wild-type *Bacillus thuringiensis* (Bt) *cry1C* gene could be expressed efficiently in the chloroplasts of transplastomic tobacco plants, resulting in high resistance to tobacco insects. A DNA fragment with the toxic domain of a wild-type Bt *cry1C* gene was constructed under the control of *rbcL* promoter and an *E. coli thra* terminator in a plasmid vector flanked by chloroplast sequences. This plasmid vector was then delivered into the chloroplast through biolistic bombardment technique. Plant transformants that possessed spectinomycin and streptomycin resistance were selected, and the integration of *cry1C* gene in these transformants was confirmed by PCR and Southern analyses. Results from PCR and Southern hybridization indicated that the truncated *cry1C* gene was integrated into the chloroplast genome as we expected through homologous recombination. High expression level of the integrated Bt *cry1C* gene in the chloroplasts was observed through Northern and Western hybridization assays. These transplastomic tobacco plants are highly toxic to the tobacco cutworm *Spodoptera litura,* causing a mortality rate of 76.9% to 100% after 72 h of feeding. The truncated Bt toxin is expressed in high levels in the chloroplast (about 1% of total proteins) and the plastid transgenes were known not to transmit through pollen. So that, this study will facilitate not only improvement in breeding for insect-resistant plants, but also the prevention of contamination of transgenes among crop plants.

Keywords: *Bacillus thuringiensis*; Chloroplast; *cry1C* gene; Insecticidal protein; *Nicotiana tabaccum*; Plastid transformation; *Spodoptera lituar*.

Abbreviations: **BA**, N^6-benzyl adenine; **BAW**, beet armyworm; **Bt**, *Bacillus thuringiensis*; **FAW**, fall armyworm; **GST**, glutathione S-transferase; **ICP**, insecticidal crystal protein; **MCCW**, Mediterranean climbing cutworm; **NAA**, α-naphthalene acetic acid; **PCR**, polymerase chain reaction; **RBS**, ribosomal binding site; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **TCW**, tobacco cutworm.

Introduction

Crystal (Cry) proteins from *Bacillus thuringiensis* (Bt) have an insecticidal effect on a number of insects. Most Bt toxins are synthesized as protoxins that are then proteolytically converted into active toxin fragments (ca. 60-70 kDa) in the insect midgut. The activated toxin interacts with the midgut epithelium cells of susceptible insects and generates pores in the cell membranes, disturbing the osmotic balance of cells. This causes the insect larvae to stop feeding and eventually die (Gill et al., 1992). Attempts to express Bt toxin genes in plants through nuclear genome transformation have been very successful (Barton et al., 1987; Fischhoff, 1987; Vaeck et al., 1987; Perlak et al., 1990; Cheng et al., 1992; Fujimoto et al., 1993; Koziel et al., 1993; Van der Salm et al., 1994; Stewart et al., 1996; Nayak et al., 1997; Cheng et al., 1998). However, the expression levels of original unmodified Bt toxin genes in plants were extremely low, in the range of 0.001 - 0.005% of total soluble proteins (Barton et al., 1987; Fischhoff, 1987; Vaeck et al., 1987). Numerous factors have been proposed as the cause of this low expression level, such as premature transcription termination, aberrant mRNA splicing, mRNA instability, and inefficient codon usage (Perlak et al., 1991). The synthetic Bt toxin genes designed to avoid many of the undesirable features and unfavorable expression in plants have resulted in dramatic increases of Bt gene expression levels, up to 0.1-0.3% of total soluble proteins (Perlak et al., 1990; Fujimoto et al., 1993; Koziel et al., 1993; Van der Salm et al., 1994; Cheng et al., 1998). However, artificial synthesis of large Bt toxin genes is laborious and cost inefficient and, therefore, has limited applications in the long run.

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It has long been known that the plastid is of prokaryotic origin and retains a similar transcription and translation system of prokaryotic cells. In addition, plant cells may contain thousands of copies of the plastid genome. Therefore, stable incorporation of a prokaryotic transgene into a plastid genome could result in the amplification of the transgene and consequently cause a much higher expression level of its protein. For example, a full-length *cry1Ac* gene with no modification was cloned into the loci between *trnV* and *rps12*/*7* of tobacco plastid genome, producing insecticidal crystal protein (ICP) at 3-5% of the total soluble proteins (McBride et al., 1995). Another full-length *cry2Aa2* gene in tobacco plastid genome gave rise to 2-4% ICP that conferred a strong insecticidal activity against insect pests (Kota et al., 1999). These cases demonstrate the expression levels of Bt toxin genes in plastid genome are much higher than those in the nuclear genome. As a result, plastid genome transformed with a wild-type Bt gene will provide a better alternative in controlling insect pests.

Unfortunately, all these successful examples in the plastid transformation used *cry1Ac* or *cry2Aa2* Bt genes as the target genes (McBride et al., 1995; Kota et al., 1999). The major insect pest, the *Spodoptera* species, shows limited susceptibility to the protein product of *cry1Ac* and *cry2Aa2* genes. The *Spodoptera* species, the polyphagous cutworms, have caused serious damage to many economic crops in the world. For instance, beet armyworm (BAW, *S. exigua*) attacks more than 90 species of at least 18 plant families throughout North America, many of which are crop plants (Greenberg et al., 2001). Fall armyworm (FAW, *S. frugiperda*) and Mediterranean climbing cutworm (MCCW, *S. littoralis*, also known as the Egyptian cotton leafworm) also cause severe crop damage in South America and Africa (Hosny et al., 1986; Hruska and Gould, 1997). In Asia, especially the Southern and Western Pacific regions, tobacco cutworm (TCW, *S. litura*) is the major insect pest (Waterhouse, 1997). In Taiwan, most of the Cruciferae family of plants and some flower species are seriously damaged by TCW or BAW.

It has been reported that the Cry1C protein is the Bt toxin in the Cry1 family with the best insecticidal activity against the BAW, TCW, and other *Spodoptera* species (Hofte and Whiteley, 1989; Visser et al., 1990; Crickmore et al., 1998). Therefore, transforming the *cry1C*-type genes into transgenic plants presents the best option for insect control, especially against the *Spodoptera* species. Several studies have used *cry1C*-type of Bt genes as targets to transform the nuclear genome for the protection against *Spodoptera* species (Van der Salm et al., 1994; Strizhov et al., 1996; Christov et al., 1999). For example, the modified *cry1Ca1* gene (GenBank accession no. X07518) and synthetic *cry1Ca5* gene (GenBank accession no. X96682) transferred into the nuclear genome of alfalfa and tobacco plants help protect them from the attack of *Spodoptera* insects, such as BAW, MCCW (Van der Salm et al., 1994; Strizhov et al., 1996), and TCW (Christov et al., 1999). However, no *cry1C*-type of Bt gene has been found in transforming the plastid genome. We report here our attempt to transform a *cry1C* gene into the plastid genome and the over-expression of *cry1C* in chloroplasts confers high insecticidal efficacy against TCW, a *Spodoptera* insect.

Materials and Methods

Gene and Plasmid Constructions

A 1,989 bp DNA fragment encoding toxic domain of a wild-type *cry1C* gene (a truncated-form of Bt gene) was amplified by polymerase chain reaction with primers PSB25 (5′-GAGATGGAGGTAACCCATGGAGGAA-3′, start codon underlined) and 28SstIC (5′-CTGGAGCTCA MTCMACTAAATTGGATAC-3′; M=A+C, stop codon underlined), and plasmid pSB744 (Chak et al., 1994) was used as the template. This amplified *cry1C* gene with *Nco*I and *Sac*I DNA fragment was cloned into plasmid pUN4 (Lin et al., 2002b) to form pUNIC (Figure 1), or into plasmid pJS25D (Lin et al., 2002a) to form pJC (Figure 1). A *Bam*HI–*Eco*RI fragment including the *cry1C* gene from pUNIC was subcloned into pGEX-3X (GST gene fusion vector, Amersham Pharmacia Biotech, Buckinghamshire, England) to form pGEX-IC, resulting in an in-frame fusion of the GST-CryIC (Figure 1). The GST-CryIC fusion protein expressed in *E. coli* was purified through GST-affinity chromatography and then used to produce antibodies for future applications.

Construction of *cry1C* gene containing vectors for plastid transformation was basically modified from the plasmid pZS197 (Svab and Maliga, 1993). The pZS197 was a transformation vector comprised sequence for homologous recombination from base 57750 to 60595 of the tobacco chloroplast genome (accession number Z00044) and a spectinomycin-resistant gene, *aad*A, with the tobacco *rrn* promoter and 3′-UTR of tobacco *psb*A gene (T*psb*A) included as well. Restriction sites downstream of the T*psbA* were modified by inserting a blunt-ended *Sal*I/*Eco*RI fragment containing kanamycin-resistant gene into *Hin*dIII site of pZS197. Excising the kanamycin-resistant gene by *Sma* I and *Hin*dIII gave the remainder of pZS197 as a vector to ligate *Hin*dIII/*Pvu*II fragment from pSU86AΔ3′ (Wu et al., 1997) to form a new construct pCHL1 (Figure 1).

A *Spe*I–*Bam*HI fragment containing a ribosome-binding site (RBS) and the *cry*1C gene from pJC was cloned into pLsTa (Chen and Orozco, 1988) to form the construct pLCTa (Figure 1), where the *cry*1C transcripts could be initiated by the promoter of spinach chloroplast *rbc*L gene and terminated by the *E. coli thra* terminator (Chen and Orozco, 1988). The *Hin*dIII/*Eco*RI fragment of pLCTa was cloned into pCHL1, giving rise to a final construct, pNIC101 (Figure 1), composed of all sequences necessary for *cry1C* gene expression in the plastid and used for plastid transformation in this study.

Plastid Transformation and Plant Regeneration

Plastid transformation and generation of homoplasmic tobacco lines was carried out essentially as described by Svab and Maliga (1993). Sixty mg of the microcarriers

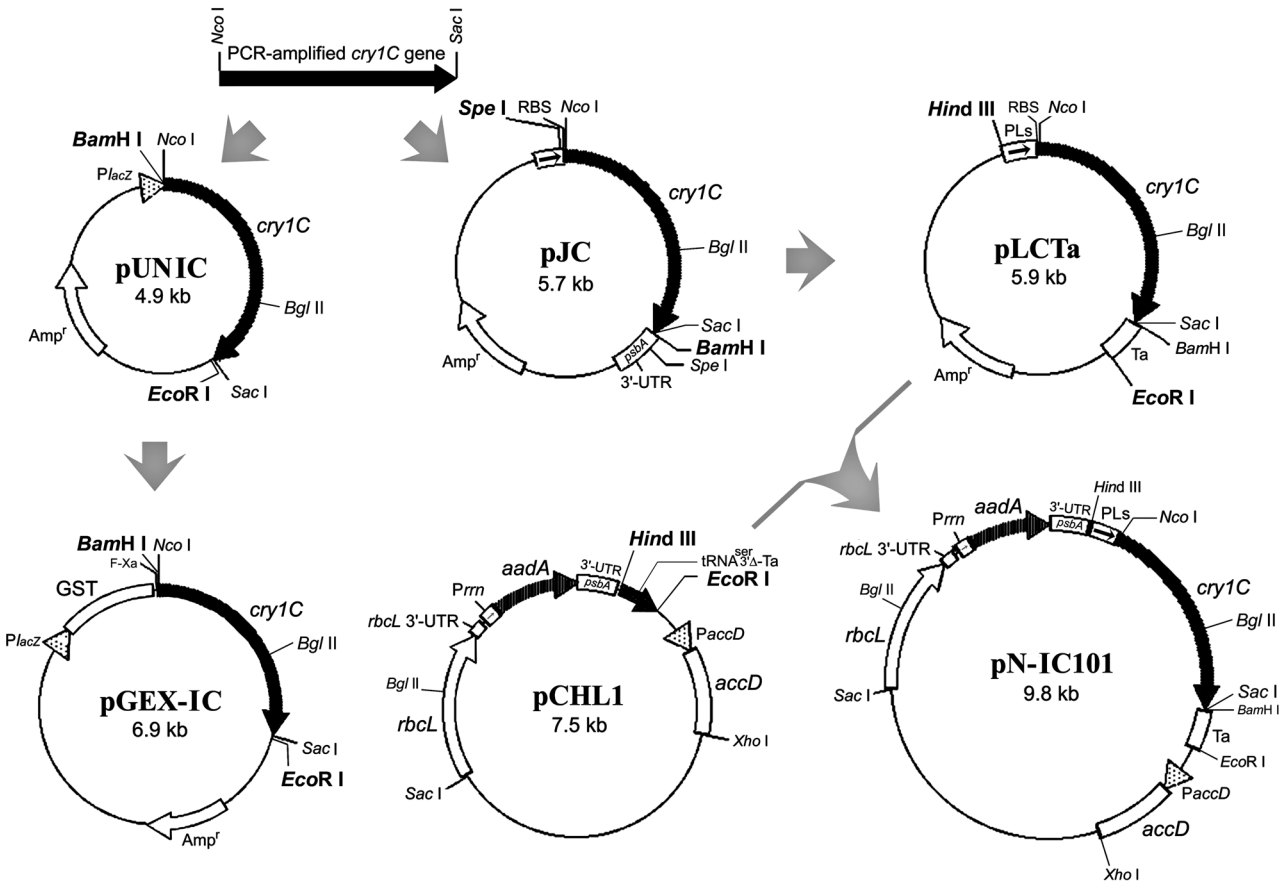

Figure 1. Construction of plasmid expression vectors. The PCR-amplified *Nco*I and *Sac*I DNA fragment containing *cry1C* gene was cloned into the pUN4 and pJS25D to form pUNIC and pJC, respectively. The *Bam*HI-*Eco*RI fragment of pUNIC was subcloned into the pGEX-3X to create pGEX-IC used for Cry1C protein purification through GST-affinity chromatography. The *cry1C* gene of the pJC was subcloned into pTZ19-PLsTa to form pLCTa, where the *cry1C* transcripts could be initiated by the promoter of spinach chloroplast *rbc*L gene and terminated by the *E. coli thra* terminator (Chen and Orozco, 1988). The *Hin*dIII/*Eco*RI fragment of pLCTa was cloned into pCHL1, which gave rise to a final construct pN-IC101, composed of all sequences necessary for *cry1C* gene expression in the plastid and used for plastid transformation in this study.

(tungsten, M-10, Bio-Rad, Hercules, CA, USA) were sterilized with 75% ethanol, then washed twice with sterilized distilled deionized water (ddH_2O). The washed microcarriers were precipitated and then resuspended in 1 ml ddH_2O and stocked at -20°C for later use. Each 50 µl of microcarrier stock can be used for three to six bombardments. For DNA coating, the 50 µl of microcarrier was vortexed mildly in an eppendorf tube, and 5 µl of the plasmid DNA (1 µg/µl), 50 µl of the 2.5 M $CaCl_2$, and 20 µl of the 100 mM spermidine were subsequently added in order. The mixture was kept on ice for 10 min, then was washed twice with 200 µl of the 75% ethanol. The supernatant was then discarded, and the DNA-coated tungsten pellet was resuspended in 20 µl of the 95% ethanol.

The commercialized particle gun (PDS-1000He, Bio-Rad, Hercules, CA, USA) was used for the bombardment. The manufacturer's procedures were followed. An aliquot of 5-µl of the prepared microcarriers was homogeneously spread on the macrocarrier disc held by the stainless holder. The 1100 psi rupture disc was set up, and the target tissue was placed on the bottom of the chamber 9 cm from the macrocarrier for bombardment. All units of the apparatuses were assembled according to the manufacturer's manual.

A 4-week-old leaf disc (*Nicotiana tabaccum* cv. Petit Havana) was bombarded in the chamber with a vacuum set at 25 inches Hg. The bombarded leaf disc was then cultured on the RMOP medium (4.32 g/L MS salts [GIBCO BRL, Life Technologies, Gaithersburg, MD, USA], 1 mg/L thiamine-HCl, 0.1 g/L myoinositol, 3% sucrose, 1 mg/L BA, 0.1 mg/L NAA, 0.4% phytagar, pH 5.8) and incubated in the dark for 2-4 days. After that, the leaf was sliced into 5-mm^2 pieces and transferred onto the RMOP medium containing 500 µg/ml of the spectinomycin. The explants were incubated at 24°C under 16:8 L:D cycle, and the medium was replaced regularly every 2 weeks to maintain the antibiotic and phytohormones in active condition. All the explants expanded and turned brown and white within 1 month. Some green adventitious shoots or calli appeared within 2 months. The green tissues were subcultured on fresh RMOP medium every 2 weeks for over 6 months. On each subculture, only the newly formed apical shoot was

cut and transferred to the fresh medium. Regenerated plants were placed on MS medium (4.32 g/L MS salts, 0.4 mg/L thiamine-HCl, 0.1 g/L myoinositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 2 mg/L glycine, 3% sucrose, 0.4% phytagar, pH 5.8), 0.1 mg/L of the NAA, and 500 µg/ml of the spectinomycin for rooting. The *cry1C* gene in regenerated plants was confirmed by PCR, and then the plants were planted in pots with autoclaved soils in the greenhouse. All were grown in the greenhouse with 100% humidity for 2 weeks or more, and then the humidity was reduced to the levels similar to the ambient environment.

DNA and RNA Blotting

Total DNA from mature leaf of non-transformed and transformed tobaccos was extracted by a DNA extraction kit (Plant Genomic DNA Extraction Miniprep System, GPG1001, Viogene, CA, USA) with no RNA contamination. Total RNA was extracted by the TRIZOL® reagent (GIBCO BRL, Life Technologies, Gaithersburg, MD, USA) according to the manufacturer suggestions.

Equal amounts of the total DNA or RNA were applied for blotting analysis. Total DNA from the assayed plants was digested with *Bgl*II, *Eco*RI, and *Sac*I, respectively. Each digested DNA was fractionated in a 1% agarose gel and then transferred onto the nylon membrane for hybridization (Sambrook et al., 1989). A 2.2-kb *Pst*I–*Xho*I fragment, as shown in Figure 1, was used as the probe. Total RNA from each sample was fractionated in a 1.2% formaldehyde agarose gel and transferred onto the nylon membrane. A PCR-amplified 598-bp fragment containing the coding sequence of the *cry1C* gene (Lin et al., 2002a) served as the probe in RNA blotting.

Immunoblotting and Quantification of Protein

Total soluble proteins were isolated from leaves of non-transformed and transformed tobaccos according to methods described previously (McBride et al., 1995). The protein concentration of each sample was measured by the protein assay reagent (BCA™ Protein Assay kit, Pierce, Rockford, IL, USA) according to the manufacturer suggestions. An appropriate amount of the soluble proteins from each sample was analyzed by SDS-PAGE with a 10% polyacrylamide gel (Sambrook et al., 1989), using the prestained protein standards (Broad Range Control #85059, Bio-Rad, Hercules, CA, USA) as marker. After electrophoresis, the gel was soaked in an ice-chilled transblotting buffer (2.5 mM Tris, 192 mM glycine, 20% methanol, 0.05% SDS, pH 8.3) for 30 min. The Semi-Dry transblotter (Bio-Rad, Hercules, CA, USA) was used to transfer the proteins onto a membrane with an electrical field of 22 Volts for 50 min. After transfer, the membrane was soaked in the TBS-milk buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5% non-fat milk powder) and shaken for 30 min. A primary antibody, the rabbit antiserum that reacts specifically against the Cry1C protein, was added in the buffer and shaken for another 2 h at room temperature. The membrane was washed five times by ice-chilled TBS buffer for 10 min, then soaked again in the TBS-milk buffer with the addition of a secondary antibody (1:5,000, v/v), the alkaline phosphatase conjugated anti-rabbit goat IgG (H+L) (Pierce, Rockford, IL, USA). The membrane was then soaked in 20 ml of the APB buffer (10 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), with the following chromogenic reagents: 132 µl of the NBT reagent (5% nitro-blue tetrazolium chloride in 70% dimethylformamide) and 64 µl of the BCIP reagent (5% 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt in 100% dimethylformamide), added in order to develop the reaction signals. The signals usually developed within 10 min and were preserved by scanning the membrane with a high sensitive scanner in logarithmic TIFF format. These signals were then digitized and quantified by the Image Gauge™ (Science Lab Image Analysis Software, Fuji Science, Tokyo, Japan). A standard curve was obtained by serial dilutions of the known amount of purified Cry1C protein. Results showed that the Cry1C protein concentration of 1-500 ng is within the linear range of the band intensity measured by the Image Gauge™.

Insect Bioassay

The 3rd-instar larvae of the tobacco cut worm (*S. litura*) were used for the insecticidal activity assay for the regenerated tobaccos and their T_1 progeny. The mature leaves of the non-transformed and transformed tobaccos were detached, and their leaf areas were measured by the LI3100 leaf area meter (Li-Cor, Lincoln, NE, USA). An average of one larva per 20 cm^2 of leaf area was applied in this assay; each tested leaf was soaked in the water to maintain its freshness. This assay was performed in a growth chamber with 25°C, 75% RH, and 12:12 L:D cycle. The dead larvae were counted in 24-h intervals, and the average weight of the larvae was measured at the beginning of the assay and at 72 h after the feeding. Three replicates of the treatment for each plant (9 to 16 larvae were used, see Table 1 for detail) were performed to estimate the mortality rate. The insecticidal activity assays for non-transformed and transformed whole tobacco plants were conducted in an open space where equivalent amounts of insect larvae were applied.

Results

Construction of a Chloroplast Expression Vector and Plastid Transformation

A plasmid pCHL1 (Lin et al., 2002a), containing the aminoglycoside 3′-adenyltransferase (*aadA*) gene (that confers resistance to spectinomycin/streptomycin) and the tobacco chloroplast DNA fragment of the *rbcL* and *accD* regions, was used as the template for constructing the chloroplast expression vector. An expression cassette containing a truncated Bt toxin gene (*cry1C*, see Material and Methods for detail) driven by the chloroplast promoter P*rbcL* and an *E. coli thra* terminator (Chen and Orozco, 1988) was cloned into the intergenic spacer region between *rbcL* and *accD*. The resulting chloroplast expression vector, pN-IC101, was able to direct the insertion of

the Bt toxin gene into the tobacco plastid genome between the *rbcL* and *accD* loci by homologous recombination (Figure 1). Expression vector pN-IC101 was introduced into the tobacco plastid genome using a biolistic process described previously (Svab et al., 1990; Svab and Maliga, 1993). Transformed plastids carrying the *aadA* gene were selectively amplified by growing bombarded leaves on a spectinomycin-containing medium. Most of the bombarded leaves were bleached at 4 weeks while a few green shoots (spectinomycin resistant) emerged from underneath the bleached leaves after 5 to 7 weeks of antibiotic selection. Seven antibiotic resistant lines were regenerated, and they were further sub-cultured in medium containing both spectinomycin and streptomycin to achieve plants with homogenous plastid genomes, the homoplasmy. Among those lines, only plants propagated from four antibiotic resistant lines, IC-5, -8, -13 and -16, were selected for further studies.

Screening and Identification of Homogenous Transplastomic Plants Containing cry1C Gene

Initially, these four antibiotic resistant plants were screened using PCR to detect the existence of *cry1C* gene in the chloroplast genome. Primers used in the PCR were designed to identify the 2.0-kb *cry1C* gene. The presence of a 2.0-kb PCR product in all four transgenic lines was observed whereas no PCR product was detected in the wild-type tobacco (data not shown). After a minimum of five subcultures of these four transgenic lines in the presence of antibiotics, they were further analyzed by Southern hybridization to evaluate the site-specific integration of *cry1C* gene and the degree of homoplasmy in their plastid genomes. The structure of the wild-type (WT) plastid genome with the expected foreign genes *aadA* and *cry1C* integrated into the transformed plastid genome (Nt-IC), and the various lengths of restriction fragments when hybridized by the 2.2-kb *Pst*I-*Xho*I probe were shown (Figure 2A). The Southern hybridization patterns with *Bgl*II, *EcoR* I and *Sac* I enzymes for these four transgenic lines were the same as predicted (Figure 2B). The integration of the *aadA* and *cry1C* gene expression cassettes in the plastid genome introduced an additional restriction site for each of the *Bgl* II, *EcoR* I and *Sac* I enzymes. This resulted in a 3.4 kb (a faint band due to less sequenced homology hybridized to the probe, indicated by arrowhead in Figure 2B) and a 3.1 kb in transgenic plastid genome (Nt-IC) instead of a 2.7 kb in the wild-type genome (WT) with the *Bgl*II digestion. Similarly, the 4.4-kb *Eco*RI fragment in WT was replaced by a 5.5-kb and a 2.7-kb in the Nt-IC, and the 10.7-kb *Sac*I fragment in WT was replaced by a 6.5-kb and a 7.8-kb in the Nt-IC (Figure 2). These results demonstrate clearly that the *cry1C* gene was site-specifically integrated between *rbcL* and *accD* genes of the tobacco plastid genome (Figure 2). It has been known that each plant cell contains multi-copies of chloroplast genomes, and by analyzing the hybridization patterns of Southern blots of these four transgenic lines, it was confirmed that these transgenic lines are in complete homoplasmy, with no wild-type chloroplast genomes remaining in the cell. We therefore predict the copy number of *cry1C* genes will equal to the number of the plastid genomes in the cell.

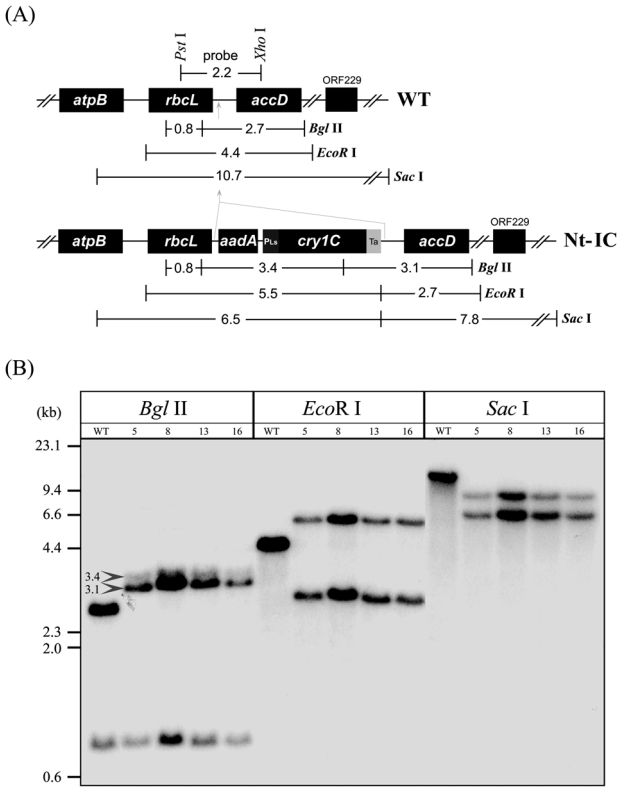

Figure 2. DNA gel blotting assay of transplastomic tobaccos. (A) Schematic diagram of the *rbcL* and *accD* genes and their flanking regions in the plastid genome of the non-transformed (WT) and the transformed (Nt-IC) tobaccos. The transformed (Nt-IC) plastid genome contains the *aadA* and *cry1C* genes fragments, as shown in the construct pN-IC101, integrated into the region of the WT plastid genome (indicated by the arrow) by homologous recombination. The various lengths of restriction fragments expected to be hybridized by the 2.2-kb *Pst*I-*Xho*I probe are shown. (B) Plastid genomes of four independent transplastomic Nt-IC-5, Nt-IC-8, Nt-IC-13, Nt-IC-16 and the non-transformed tobaccos were examined by the DNA gel blot. Total DNA from these tobaccos were digested with *Bgl*II, *Eco*RI, and *Sac*I, respectively, and hybridized by the 2.2-kb *Pst*I-*Xho*I probe indicated in (A). The hybridization signals were developed by autoradiography, and their sizes in kb were estimated based on the l DNA/*Hin*dIII DNA marker as indicated on the left. Lanes 5, 8, 13, and 16 represent the four individual transgenic plants IC-5, -8, -13, and -16, respectively.

Expression of cry1C Gene in Transplastomic Plants

To test for production of *cry1C* mRNA in these four transplastomic tobacco lines, RNA gel blot analysis was performed (Figure 3A). A strong signal of approximately 2.5 kb was detected for all four transplastomic tobacco lines using *cry1C* gene probe while no signal was revealed in the control plant (Figure 3A, top panel). The expressions of 16S rRNA were used as internal controls to dem-

onstrate that an equal amount of RNA in each sample was loaded (Figure 3A, bottom panel).

To confirm and quantify the protein expression of *cry1C* gene, total soluble protein extracted from transformed and non-transformed leaves were subjected to Western blot analysis. A single protein fragment with apparent molecular weight of 70 kDa was observed in all four transformed tobacco lines, but no protein was detected in the non-transformed plant (Figure 3B). The transgenic line IC-5 showed degradation of its Cry1C protein while the other three lines did not. The abundance of the Cry1C protein in transgenic line IC-5 and its T_1 progeny was estimated using the known amount of Cry1C proteins running parallel in Western blotting gel. Band intensity of 10 ng Cry1C in Figure 3C and of 2 ng Cry1C in Figure 3D were set as the value of 1, and each tenfold increment of known Cry1C was measured to set the titration curve. The relative intensity of protein samples was measured and shown at the bottom of each sample (Figure 3C and D). The relative intensity (76.7 instead of 100) of the 1 µg Cry1C was beyond the linear range of quantification. We therefore reestablished a more accurate standard curve for calculating the protein sample. In this instance, about 290 ng of Cry1C protein, equal to 1.1%, in the total soluble proteins (25 µg) of transgenic line IC-5 was estimated. T_1 progeny of the IC-5 plant (IC-5-T_1) was measured in the same way. The estimated amount of Cry1C in IC-5-T_1 plant was approximately 4% of the total soluble proteins. The large amount of Cry1C protein produced in transplastomic plant led us to predict that a significant insecticidal activity against the *Spodoptera* species could be assured.

Insecticidal Activity Assays

To test the toxicity of various transplastomic lines to insect, a single leaf was detached and whole plant assays from each line were conducted. Larvae of the tobacco cut worm (*S. litura*) were used in insecticidal activity assays. One 3rd-instar larva per 20-cm^2 leaf area was applied to each replicate. Data was obtained from each of the three replicates (Table 1). Results in Table 1 showed that

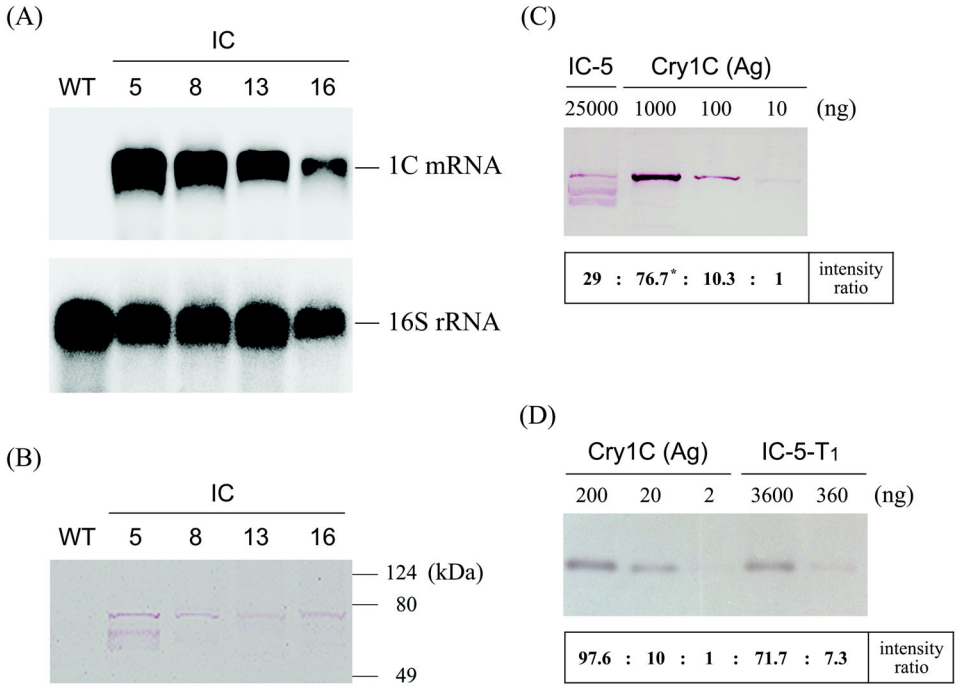

Figure 3. RNA and immuno blotting assays for *cry1C* gene expression in transplastomic tobaccos. (A) The total RNAs were isolated by LiCl precipitation and fractionated in the formaldehyde gel. Equal amounts of RNA samples were loaded. Filters were duplicated for different probes hybridization. The top panel of (A) shows the *cry1C* transcripts probed by *Nco*I / *Bgl*II *cry1C* fragment. The bottom panel of (A) is the chloroplast 16S rRNA transcripts. Lane WT in each panel is an RNA sample from the wild-type tobacco. Lanes 5, 8, 13, 16 are RNA samples from transplastomic Nt-IC-5, Nt-IC-8, Nt-IC-13, Nt-IC-16 tobaccos, respectively. (B) Total proteins isolated from mature leaves of the non-transformed (WT) and the transformed (Nt-IC-5, -8, -13, -16) tobaccos were subjected to SDS-PAGE and then transferred to PVDF filter for immunoblot assay. The Cry IC proteins with an apparent molecular mass of 73 kDa were detected as described in Materials and Methods and shown in panel (B). Degradation of Cry1C protein from Nt-IC-5 was observed. (C) Twenty-five µg (25,000 ng) of the total soluble proteins from a mature leaf of the IC-5 plant and 1000, 100, and 10 ng of the purified Cry1C protein were analyzed. The signal intensities of IC-5 (including degraded products) were calculated by Image Gauge™ v. 3.1 (Fuji Science, Tokyo, Japan) based on the standard curve obtained from known amounts of Cry1C protein in series dilution. The signal intensity of 10 ng of Cry1C protein was set as 1; the relative intensity ratio for each sample was then estimated and shown in the bottom of panel (C). The asterisk indicating the 1000 ng of Cry1C is out of the linear range of calculation. (D) The same calculation method in (C) was applied to T_1 progeny of Nt-IC-5, except that the amount of each protein sample was adjusted to coincide within the linear range of calculations.

Table 1. Insecticidal efficacy of the *cry1C* gene-transformed transplastomic tobaccos against the larvae of tobacco cut worm (*Spodoptera litura*)[a]

Plants	n[c]	Mortality (%)[b]			Larvae average weight (mg)[f]	
		Time after treatment (hours)			Time measured (hours)	
		24 h	48 h	72 h	0 h	72 h
IC-5	14	0.0	57.1	100.0	3.57	2.50
IC-8	13	7.7	30.8	76.9	3.57	1.57
IC-13	13	0.0	53.8	100.0	3.47	2.03
IC-16	9	22.2	33.3	100.0	3.47	2.67
IC-5-T_1[d]	16	56.3	100.0	-	-	-
WT[e]	15	0.0	0.0	0.0	3.03	36.3

[a]The 3rd-instar larvae were used.
[b]An average of one larva per 20 cm^2 of leaf area and 3 replicates for each plant were applied in this study. Tested leaves were soaked in water to keep fresh.
[c]Number of insect larvae used.
[d]Progeny of the regenerated IC-5 plant.
[e]WT, the wild-type tobacco.
[f]Weight of larvae were measured at 0 and 72 h after treatment.

transplastomic lines IC-5, IC-13 and IC-16 resulted in a 100% mortality after 72 h of feeding while a 76.9% mortality for line IC-8 was observed (Table 1). All the insect larvae tested began with an average weight of 3.5 mg, and the dead larvae weighed in at 1.6 to 2.7 mg after 72 h of feeding with transformed tobacco leaves whereas the weight of larvae feeding with non-transformed tobacco leaves increased 12-fold (Table 1). This data indicated that unmodified truncated Bt *cry1C* gene in plastid genome confers high insecticidal efficacy against tobacco cut worm. Representative photographs taken from transplastomic lines IC-5, IC-13 for single leaf assay (Figure 4A) and line IC-5 for whole plant assays (Figure 4B) are shown. The entire leaves of wild-type control were almost completely consumed while little detectable feeding damage was observed for the transplastomic line IC-5 (Figure 4A, B).

Germination and Insecticidal Activity Assays of T_1 Progeny

T_1 seeds of transplastomic line IC-5 were harvested, and their germination against antibiotic and insecticidal activity against TCW was analyzed. Seeds from different capsules of transplastomic line IC-5 were sown on sterilized MS medium containing 500 μg/ml of the spectinomycine (Figure 4C-a, b), and seeds from wild-type tobacco were sown on the same MS medium as well with (Figure 4C-d) or without spectinomycine (Figure 4C-c) for comparison. All transplastomic T_1 seeds germinated and showed resistance to antibiotics (Figure 4C-a, b). In contrast, the WT seeds showed sensitivity to the antibiotic, as the entire seedling bleached out in antibiotic-containing MS medium (Figure 4C-d) while remaining green in antibiotic-free medium (Figure 4C-c). This observation indicates that our T_0 transgenic plants were in homoplasmy and the *aadA* transgene was physically linked to the *cry1C* gene, as no segregation between them was observed. T_1 progeny from transplastomic line IC-5 was further transferred to pots in greenhouse under conditions where no antibiotic was applied, to test its insecticidal activity against TCW. As shown in Table 1, detached mature leaves from the progeny plant (IC-5-T_1) revealed 56% mortality against TCW at 24 h and 100% 48 h after treatment. The better performance of the T_1 plant relative to its ancestor in insecticidal efficacy could possibly be correlated to the higher expression level of Cry1C protein in T_1 plant (4%) than in T_0 (1.1%) (Figure 3D). A representative photograph showing the result of bioassay from T_1 progeny of line IC-5 is given (Figure 4D).

Discussion

Both the Truncated cry1C Gene and the E. coli thra Terminator Were Well Recognized in Plastid Genome

It has been reported that nuclear transgenic plants transformed with truncated N-terminal Bt toxin genes could achieve higher levels of Bt toxin than those transformed with intact genes (Vaeck et al., 1987). Although several studies have transformed plastid genome with Bt toxin genes for insect control (McBride et al., 1995; Kota et al., 1999; Zhang et al., 2000; De Cosa et al., 2001), the Bt toxin genes used were all intact genes with both the N-terminal toxic domain and the C-terminal crystal-forming domain. No indication of truncated N-terminal Bt toxin gene was used in previous studies. In this approach, we introduced a truncated *cry1C* gene into the plastid genome, and the transformed transplastomic plants showed a high level of Cry1C production (Figure 3), with the amount comparable to those using intact Bt genes (McBride et al., 1995; Kota et al., 1999; De Cosa et al., 2001). We therefore suggest that the truncated-type of Bt toxin gene is an appropriate form of Bt gene for expression in plastid genomes. This provides an alternative version of application of Bt toxin gene in transplastomic plant studies.

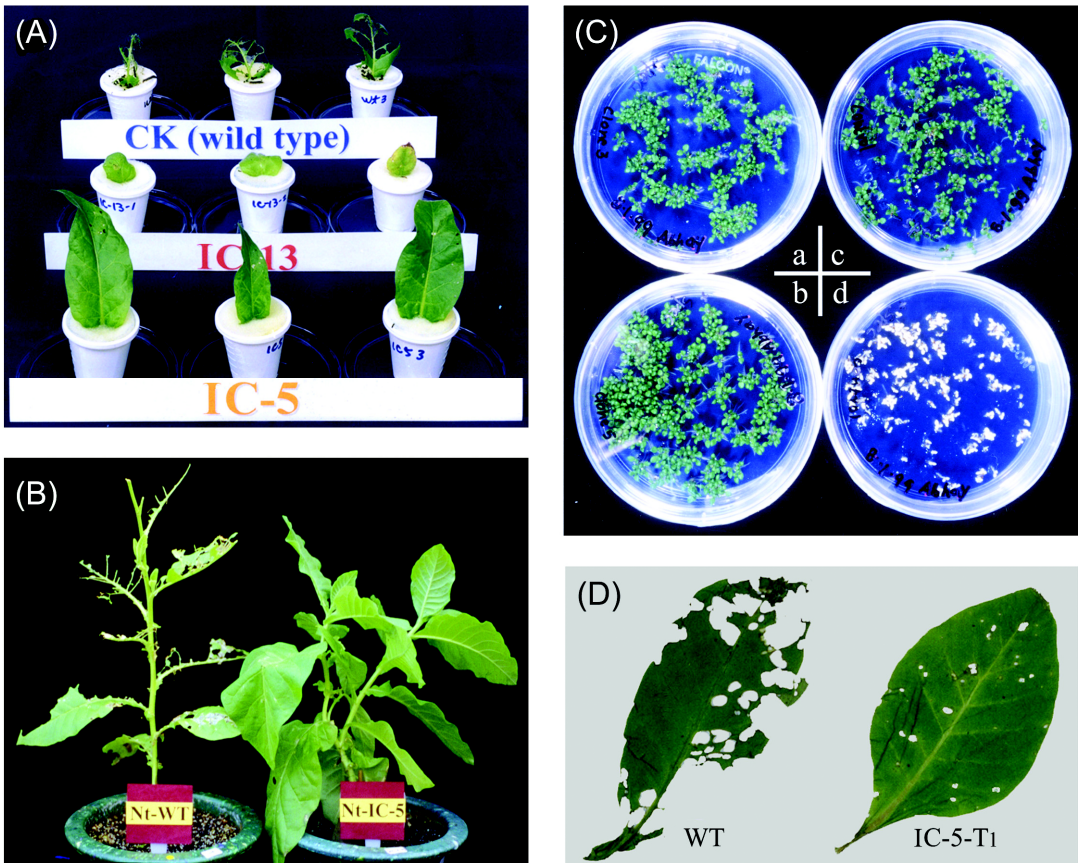

Figure 4. Insecticidal activity assay of transplastomic tobaccos and T_1 seed germination test against antibiotic. (A) Single leaves detached from two transgenic lines, IC-5 and IC-13, were conducted for the insecticidal activity against TCW (*Spodoptera litura*). Non-transformed leaves were used as control (CK, wild type). One 3rd-instar larva per 20 cm^2 leaf area was applied to each replicate. (B) Insecticidal activity using whole plants from the transgenic IC-5 (Nt-IC-5) and the wild-type (Nt-WT) tobaccos were performed by the feeding of 3rd-instar larvae of TCW, and the photograph of this experiment was taken 72 h after feeding. (C) Antibiotic-resistant T_1 seed germination was analyzed in medium containing streptomycin (500 µg/ml). Seeds from different capsules of the IC-5 plant were sterilized and sown on MS medium only (a) or MS containing 500 µg/ml of streptomycin (b). Non-transformed seeds sown on MS medium with (d) or without (c) streptomycin were used as controls. (D) Single leaf detached from T_1 progeny (IC-5-T_1) and non-transformed tobacco (WT) was treated as that in (A). Representative photographs in (A), (B) and (D) were taken 72 h after feeding.

In addition, we first introduced the *E. coli thra* terminator (Chen and Orozco, 1988) in our vector construct and demonstrated its function in plastid genome. The high level of mRNA accumulation in the transplastomic plants (Figure 3A) indicates that the *thra* terminator used in this Bt vector construct is functional and also implies that the *thra* terminator can stabilize mRNAs in the chloroplast. This observation suggests that, although indirectly, the *E. coli thra* terminator was well recognized by the chloroplast transcription machinery and may play a significant role in terminating transcription and stabilizing mRNAs in the plastid genome as well.

The Wild-Type cry1C Gene Integrated in Plastid Genome Resulted in High Level of Cry1C Production in Chloroplast

To date, all commercialized and most reported insect resistant transgenic crops use modified Bt toxin genes to transform their nuclear genomes (Perlak et al., 1990, 1993, 2001; Fujimoto et al., 1993; Van der Salm et al., 1994; Stewart et al., 1996; Nayak et al., 1997; Cheng et al., 1998). These genes eliminate all the undesirable features that affect the expression of Bt genes in plants, resulting in up to 100-fold increases in the total soluble proteins (Perlak et al., 1991; Fujimoto et al., 1993; Van der Salm et al., 1994; Iannacone et al., 1997; Cheng et al., 1998). Although the modified Bt genes showed a high level of expression in the cell, modification is laborious and cost inefficient, thus limiting the application of novel Bt genes. In contrast, with no modification, the wild-type Bt toxin genes used to transform the prokaryotic-like plastid genome could ensure their proper expression and result in a high-copy-number of target genes in each cell as well. It has been reported that transforming wild-type Bt toxin genes in plastid genome could reach 2-5% of Bt toxin production, about 10-50 fold more than transforming modified genes in a nuclear genome (McBride et al., 1995; Kota et al., 1999; De Cosa et

al., 2001). In this study, the same high level of Cry1C production as described in previous reports (McBride et al., 1995; Kota et al., 1999) was observed in our transplastomic plants (Figure 3D). As a result, these transplastomic plants possess high insecticidal efficacy against tobacco cut worm (Figure 4 and Table 1). The high expression level of Cry1C in transplastomic plants may have resulted from the following facts: 1) chloroplasts are prokaryotic compartments inside eukaryotic plant cells, 2) *cry1C* gene has a similar codon composition to chloroplast genes, and 3) each cell contains a high copy number of *cry1C* genes. These unique phenomena enable the use of wild-type *cry1C* gene, and a high expression level of such genes in the chloroplast is expected. Therefore, our data suggest that the plastid transformation system using truncated wild-type Bt toxin gene offers a great advantage in insect control.

Production of Bt Toxin in Plastids Provides not Only High-Dose-Strategy Insect Control, but Also the Prevention of Transgene Spread through Pollen

Using transgenic techniques for insect management has been very successful (Perlak et al., 1993, 2001; Fujimoto et al., 1993; Koziel et al., 1993; Stewart et al., 1996; Nayak et al., 1997; Cheng et al., 1998). However, the buildup of insect resistance is still the major concern for sustainable insect control. To reduce the insect resistance to transgenic Bt plant, several strategies have been suggested (McGaughey and Whalon, 1992; Estruch et al., 1997; Shelton et al., 2000). These include: 1) a high-dose strategy that increases high Bt expression levels; 2) gene pyramiding that express multiple toxins in one plant; 3) tissue-specific expression that expresses the protein only in tissues highly sensitive to damage. In this study, the high level of Cry1C toxin produced in plastids is well suited to the high-dose strategy in which all target insects are less likely to escape. So, a strategy combining the transplastomic plants showing nearly 100% mortality against insects (Table 1) with the non-transformed plants used as refugees would ensure the appropriate insect management (Shelton et al., 2000).

Plastid transformation that uses two flanking sequences, through homologous recombination, enables the insertion of foreign DNA into the spacer region between the functional genes of the plastid genome, thus targeting the foreign genes to a precise location. Such precise targeting eliminates the "position effect" frequently observed in nuclear transgenic plants. This explains why no gene silencing or position effect variation occurred in our T_1 transplastomic plants (data not shown).

Moreover, the plastid genome in most plants is inherited maternally. It is known that uniparental plastid gene inheritance prevents pollen transmission of foreign genes. Therefore, the *cry1C* gene introduced into plastid genomes could avoid or reduce the potential out-crossing that would transmit *cry1C* gene to other plants and significantly impact the natural environment.

In addition, the sequestration of Bt toxin proteins in the chloroplast would prevent the adverse interactions with the cytoplasmic environment. It is also probable that a cluster of plastid genes could be expressed as a polycistronic message by a single promoter in chloroplast (De Cosa et al., 2001; Daniell and Dhingra, 2002). All the merits mentioned above infer that chloroplast is a feasible platform for genetic modification of plants (Heifetz, 2000; Bock, 2001; Daniell et al., 2002). However, the troubles encountered using plastid transformation were the low efficiency of plastid transformation and the fact that the transformation system was only available for a few plant species, such as tobacco, tomato (Ruf et al., 2001) and potato (Sidorov et al., 1999) and not for the other major crop plants (Maliga, 2003). This limitation has restricted and delayed its application. However, the foreign gene in the plastid genome has been very stable for at least two generations as reported previously (Zoubenko et al., 1994).

In conclusion, this report demonstrates that a truncated wild-type Bt toxin gene is suitable for high expression in chloroplast and the truncated *cry1C* gene we isolated was effective against TCW. In addition, the study demonstrates that the *E. coli thra* terminator was well recognized in the chloroplast. The prokaryotic origin of chloroplast ensures the high level of expression of wild-type Bt toxin gene and provides a high dose strategy to prevent the buildup of insect resistance. The maternal inheritance feature will prevent the spread of transgenes through pollen, a public concern, and transgenes in the chloroplast also result in tissue specificity occurring predominantly where functional plastids are present. All these unique features and results provided in this study confirm that plastid transformation is an effective system for the introduction of truncated wild-type Bt toxin gene for insect control.

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蘇力菌野生型毒素基因 *cry1C* 在菸草葉綠體中之表現對斜紋夜盜蟲具高效殺蟲力

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利用葉綠體基因轉殖技術，將野生型之蘇力菌毒素蛋白基因 *cry1C* 的毒蛋白片段轉入菸草葉綠體之基因體中，可有效表達毒蛋白，並使此等葉綠體轉殖菸草具有抗蟲之效果。本研究首先選殖含有 Cry1C 毒蛋白部份之 DNA 片段，構築在含有葉綠體 *rbc*L 啟動子及一大腸桿菌終止子之中間，形成一可在葉綠體表達毒蛋白的載體。利用基因槍法及同質序列重組機制，將載體送入葉綠體基因組中。轉殖株透過抗生素篩選後，由 PCR 及南方墨點分析法確認，並利用北方及西方墨點分析法證明轉殖株可大量表現 *cry1C* mRNA及毒蛋白。對斜紋夜盜蛾幼蟲之毒害效果分析結果，顯示不同轉殖株在餵食葉片 72 小時之後可造成 76.9～100％之死亡率。由於此等葉綠體轉殖株具有大量表現轉殖毒蛋白之能力（高達總蛋白之 1%），且轉殖在葉綠體的基因不會經由花粉散佈，所以利用本系統獲得之抗蟲轉殖植物不但可提昇作物抗蟲效果，也可避免轉殖基因隨花粉散佈，造成其他非目標作物之基因污染。綜言之，利用葉綠體轉殖技術，能在植物體內有效表達不經改造之蘇力菌基因，不僅可達到蟲害防治之目的，而且不會因基因污染造成環境之衝擊。

關鍵詞：蘇力菌；菸草；斜紋夜盜蟲；葉綠體；毒素蛋白基因；葉綠體轉殖。