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Full Length Research Paper

Isolation and sequencing of the *cryI*C-like delta endotoxin gene from Egyptian strains of *Bacillus thuringiensis* toxic against Lepidoptera

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The aim of the present work was to explore the diversity of Bacillus thuringiensis (Bt) among local Egyptian isolates that produce parasporal crystalline inclusions. On the basis of bioactivities, nine isolates were collected from different ecological environments within Egypt. The Bt2K isolate was effective against Lepidoptera as compared to *B. thurigiensis* ssp. *kurstawi* that was used as a reference standard. The gene isolated from the strain was shown to be homologous to the Lepidoptera active *cryI*C gene from *B. thurigiensis* ssp. *ansawai* as shown by PCR and alignment of the complete nucleotide sequences (98% identical). Therefore, a *cryI*C insecticidal toxin coding gene was cloned from an Egyptian isolate of *B. thuringiensis*. Sequence analyses of the *cryI*C gene showed the absence of potential polyadenylation signal sequences within the coding region and a less biased codon usage than most *cryI*C like genes. Therefore, the gene may be a suitable candidate for expression in plants without extensive modification. This possibility was examined by subcloning the *cryI*C gene into a plant expression vector and then transferring it to tobacco and potato through *Agrobacterium*-mediated transformation. Tobacco and potato plants with a stably integrated native *cryI*C gene were completely protected from predation by Lepidoptera. It was concluded that novel insecticidal genes exist in nature that may not require extensive modifications for efficient expression in plants.

Key words: *Bacillus thuringiensis*, *cryI*C gene, plant transformation, Bt toxins, DNA sequencing.

INTRODUCTION

Naturally occurring isolates of the Gram-positive bacterium *Bacillus thuringiensis* produce crystalline inclusions during sporulation. These inclusions produced by a given isolate, are composed of a set of polypeptides and exhibit toxicity towards a select group of insects (Hofte, 1989; Choi et al., 2000; Porcar and Jua´rez-Pe´rez, 2003). The insecticidal proteins are highly specific towards the target insect, sometimes even at the species level, and are biodegradable (Lamptey et al., 1991). Since many insects cause major damage to a number of crop plants worldwide, it has been highly desirable to introduce the genes coding for the insecticidal proteins into crop plants to protect them from insect predation (Vaeck et al., 1987; Perlak et al., 1990; Lin et al., 2007).

Crystal (Cry) proteins from *B. thuringiensis* (Bt) have an insecticidal effect on a number of insects (Gill et al., 1992). There are nine groups of protein recognized as CryI-IX. The CryI proteins alone form several alloprotein subgroups: A(a), A(b), A(c), B, C, D, E, F, G. They are all bipyramidal crystals of 130-138 Kd effective against lepidoptera larvae. Like most Bt toxins they are

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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 makes the insect larvae to stop feeding and eventually die.

Attempts to express genes encoding Bt toxins in plants through nuclear genome transformation have been very successful (Barton et al., 1987; Fischhoff, 1987; 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 of original unmodified Bt toxin genes in plants were extremely low, in the range of 0.001 to 0.005% of total soluble proteins (Barton et al., 1987; Fischhoff, 1987; Vaeck et al., 1987). Consequently, although a number of crop plants were initially transformed with native *cry*1A genes, the transgenics offered only partial protection against the insect pests (Vaeck et al., 1987).

Numerous factors have been proposed as the cause of this low expression, such as premature transcription termination, aberrant mRNAsplicing, mRNA instability and inefficient codon usage (Perlak et al., 1991). Further, *B. thuringiensis* DNA has a low GC content as compared to plant genes. Finally, the toxin-coding genes are highly AT-rich and usually contain potential eukaryotic message destabilizing signals such as AATAAA,AATAAT or ATTTA (Murray et al., 1991; Perlak et al., 1991; Shaw and Kamen, 1986).

These sequences were believed to be recognized as polyadenylation signal sequences when expressed in plants and resulted to mRNA degradation which was the primary reason for the low expression of these genes. A well-designed set of experiments (Perlak et al., 1991) demonstrated that the presence of rare plant codons in the insecticidal toxin coding genes was not primarily responsible for the observed low level of protection in transgenic plants. Thus, the low level of protection observed in transgenic crop plants was inferred to be primarily due to low abundance of *cry* transcripts and their rapid degradation.

The abundance of transcripts of *cry* genes in transgenic plants was enhanced by partial or complete modification of the toxin coding region. These modifications were specifically engineered to (1) reduce or completely eliminate potential destabilizing sequences and (2) optimize codons for an efficient expression in plants (Fujimoto et al., 1993; Perlak et al., 1991; Strizhov et al., 1996). The synthetic Bt toxin genes were designed to avoid many of the undesirable features and unfavorable expression in plants while maintaining the same amino acid sequences they encode. They have resulted in dramatic increases of Bt gene expression, up to 0.1 to 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). These modifications resulted in an enhanced protection against insects in several plant species. However, artificial synthesis of many large Bt toxin genes is laborious and cost inefficient and, therefore, has limited applications in the developing world.

Natural variation among *B. thuringiensis* strains and the toxin proteins they encode encompass a wide phylogenetic distance. To characterize cry gene content variation among new *B. thuringiensis* strains by PCR-RFLP, many different primers may be used (Beron et al., 2005) since RFLP analysis alone cannot detect those new cry genes with the variations outside restriction sites. Beron et al. (2005) designed five degenerate primers for the detection of new cry genes from *B. thuringiensis* strains based on a two-step PCR approach. In the first step, one of the primer pairs was used to amplify the DNA fragments encoding protein regions that include consensus domains of representative proteins belonging to different Cry groups I to IX. A second PCR uses the first-step amplification products as DNA templates and the set of five primer combinations. Cloning and sequencing of the last-step amplicons allow the identification of both known and new *cry*–like genes that may encode cry proteins encompassing a wide phylogenetic distance. One hundred and twenty one *B. thuringiensis* isolates were tested against *Plutella xylostella* larvae and the high effective isolates in terms of high toxicity and UV tolerance was selected (Thaphan et al., 2011). Herein an insecticidal protein coding gene from a local isolate of *B. thuringiensis* (2K) was characterized. The native *cry*Ic gene was used to generate transgenic plants without any modification. Plants were protected against insect pests which may be comparable to those obtained with fully modified *cry*IA(b) or *cry*IA(c) genes.

MATERIALS AND METHODS

B. thuringiensis strains 1K, 2K, 3A, 4A, 5I, 6I, 7Y, 8Y, 9 and 9D were kindly provided by Dr. Shereif, Ain Shams University, Cairo, Egypt. Each was from a sample from local Egyptian area and was isolated from a soil sample. *Bacillus* sp. strains from each sample were selected as source materials for gene isolation studies.

Screening for new cry genes

DNA extraction

A fast DNA extraction procedure was adapted from Bourque et al. (1993), Kalman et al. (1993) and Xavier et al. (2007). *B. thuringiensis* colony on an overnight nutrient agar plate was transferred to a 0.5 ml microfuge tube containing 100 µl of lysis buffer (10 mM Tris- pH 8.0, 1mM EDTA, 1% Triton X-100) and kept on a boiling water bath for 10 min. The tubes were centrifuged at 14,000 xg for 5 min and the supernatant solution containing the total DNA was used as PCR template.

Identification, cloning and sequencing of the novel *cry* Ic gene

Molecular cloning techniques employed in this work were

Table 1. Specific primer pairs used for the identification of *cry* genes.

Gene	Forward primer (5'-3')	Product size (bp)	Reverse primer (5'-3') I(-)
*cry*1Aa	TTCCCTTTATTTGGGAATGC	1200	MDATYTCTAKRTCTTGACTA
*cry*1Ac	GGAAACTTTCTTTTTAATGG	1350	MDATYTCTAKRTCTTGACTA
*cry*1C	ATTTAATTTACGTGGTGTTG	1400	MDATYTCTAKRTCTTGACTA

performed. All oligonucleotides used in this work were purchased from (AccuOligo, Ebersberg, Germany). All amplifications were performed using Dream Taq DNA polymerase, Fermentas, St. Leon-Rot, Germany. The cry Ic gene was amplified by PCR using the oligonucleotides crylcF 5'ATTTAATTTACGTGGTGTTG3') and crylcR (5'MDATYTCTAKRTCTTGACTA-3') which were designed from the published *cry*Ic gene sequence (GenBank accession 2.1) (Juarez-Perez and Ferrandis, 1997).

Amplification was performed using an Eppendorf (Hamburg Germany) Mastercycler thermal cycler with the following program: a 3-min denaturation step at 95°C; 30 amplification cycles of 1 min at 95°C, 1 min at 45 to 50°C, and 1 min at 72°C; and a final extension step of 10 min at 72°C. The amplified fragment was cloned into the vector pSC (Fermentas) according to the manufacturer's instruction and sequenced (Geospzia, Seattle, WA, USA).

The plasmid containing the *cry*Ic gene (pSC-cry Ic) was extracted using the Wizard mini-preparation kit (Promega, Madison WI, USA). The plasmid DNA was digested with both *Bam*HI and *Xba*I, *Bam*HI and *Xho*I and a 1,200 bp fragment was separated by electrophoresis in an 1% (w/v) agarose gel, eluted from the gel (Gene JET Gel Extraction Kit, Fermentas) according to the manufacturer's instruction.

The purified fragment was ligated into the vector pBI121 and pET29a previously digested with *Bam*HI, *Xba*I, and *Bam*HI, *Xho*I respectively, producing the recombinant plasmids.

Transformation and plant regeneration

Generation of transgenic potato and tobacco plants

The *crylC* ORFs were cloned independent plant transformation vectors. The *crylc* from local isolate Bt2Kwas cloned in *pBI121* binary vector (Clontech, CA, USA) to give rise to *pBI- crylC*, where the *crylc* and the reporter *uidA* genes are kept under the control of separate *CaMV 35S* promoters with *npt II* as the selectable marker. The *crylcst* gene isolated from a standard isolate was cloned in *pBI121* as *BamH*I and *Xba*1 fragment with *nptII* as the selectable marker to get *pBI-crylcst*. In this case, *crylC* and the reporter gene *gfp*:*gusA* were driven by a single *CaMV 35S* promoter; however, a stop codon was inserted between the *crylc* and the reporter gene to avoid translational fusions. Importantly, different selectable markers for *crylc* gene constructs were used to aid the screening of independent single-gene transformants and double transgenics.

For plant transformation, the recombinant plasmids were transferred into *Agrobacterium tumefaciens* (LBA4404) made competent by the liquid nitrogen freeze-thaw method. Potato (*Solanum tuberosa*) and tobacco (*Nicotiana tabaccum* cv 'Petit Havana') leaf discs were transformed with either *crylc* or *crylcst* genes independently, or by transferring both *crylc* genes into transgenic plants so as to get the double transformants. The single-gene transformants were selected on either kanamycin or hygromycin, whereas the double transformants were selected on a mixture o f kanaycin (50 mg/L) and h yg roycin (2 5 mg/L) .

RESULTS AND DISCUSSION

Three pairs (direct and reverse) of universal primers were designed to detect genes by the sizes of their PCR products (Table 1). The DNA of each *B. thuringiensis* isolate served as the template in two reactions (Figure 1). (i) Reaction 1 was done with a mixture of two pairs, I(-) and I(+) (to detect 3 genes from the *cryAa*, *cryAC* and *crylC* group genes from the *B. thuringiensis* local isolate. (ii) Reaction 2 was done with a mixture of three other pairs, I(-) and I(+) with specific primer cry gene (to screen for presence of this gene in the local Egyptian strain and compare the cry genes with the standard strains (Figure 1).

For an extended PCR analysis for cry-type genes, we used the local Egyptian strain which was previously reported as a promising strain in this field, and our PCR was performed with standard *B. thuringiensis* strains with universal.

The genomic DNA and plasmid DNA of *B. thuringiensis* from different strain isolate served as the template in the reactions. The result shows that both chromosome DNA and plasmid DNA of *B. thuringiensis* Egyptian isolate reacted positively to the agarose electophoresis gel showing the expected size (1.2 kb). The specific primers designed by Juarez-Perez et al., (1997) were used to identify 3 genes from the three *cry* groups, *cry*1Aa*, cry1Ac* and *cry*1C genes (Table 1). The results show different *cry* gene profiles in chromosomal DNA of *B. thuringiensis* genes of *cry*1 (namely *cry*1Aa, *cry*AC and *cryl*c).

The PCR products were cloned, sequenced and subjected to BLAST search at NCBI. Chromosomal DNA and plasmid DNA contained different *cry* genes. In its chromosome, *cry*1Aa*, cry1Ac* and *cry*1C genes consisted of 1,200, 1,250 and 1,400 kp, respectively. The sequences of *cry*1Aa*, cry1Ac* and *cry*1C genes PCR products cloned were not in *cry* gene groups in database nucleotide sequence of cry IC gene (GenBank accession no: x96682.1). Among 10 field-isolated *B. thuringiensis* strains, the DNA of 4 strains did not amplify the universal primers. The rest were grouped in three *cry*-type gene

Figure 1. Nucleotide sequence of cry IC gene (GenBank accession no: x96682.1).

profiles.

Isolation and sequencing of a cry-IC gene coding for cry-IC proteins from local *B. thuringiensis* strains

The specific primers (labeled cryICF and cryICR) designed to identify 2 genes from the *cryIC* groups, were selected from highly variable regions in the respective genes (Table 2). The PCR analysis for the *cryIC* group, based on the primers' design (Kalman et al. 1993), was thus limited to identify two (cryICeg and cryICst) of the 2 known genes that are identified (Figure 1).

All except the two standard *B. thuringiensis* strains containing *cry* were found to also contain both *cryIC* and *cryIC* Stander of local *B. thuringiensis* subsp. *Thuringiensis* was negative to some *cry* genes, and *B. thuringiensis* subsp. *aizawai* was positive for most of the

Table 2. Specific primer pairs used for the identification of *cry*1C genes.

Gene	Forward primer (5′- 3′)	Product size (bp)	Reverse primer (5′- 3′)
*cry*1C	1C ATTTAATTTACGTGGTGTTG **1C(F,L)** ATGGAGGAAAATAATCAAAATCAAT GC **1C(F,R)** ACATGCGAAGCGACTCAGCG	1400	I(-) MDATYTCTAKRTCTTGACTA **1C(R,L)** CCTCTGACTACAGAGGTACC **1C(R,R)** TTATTCCTCCATAAGGAGTAATTCC

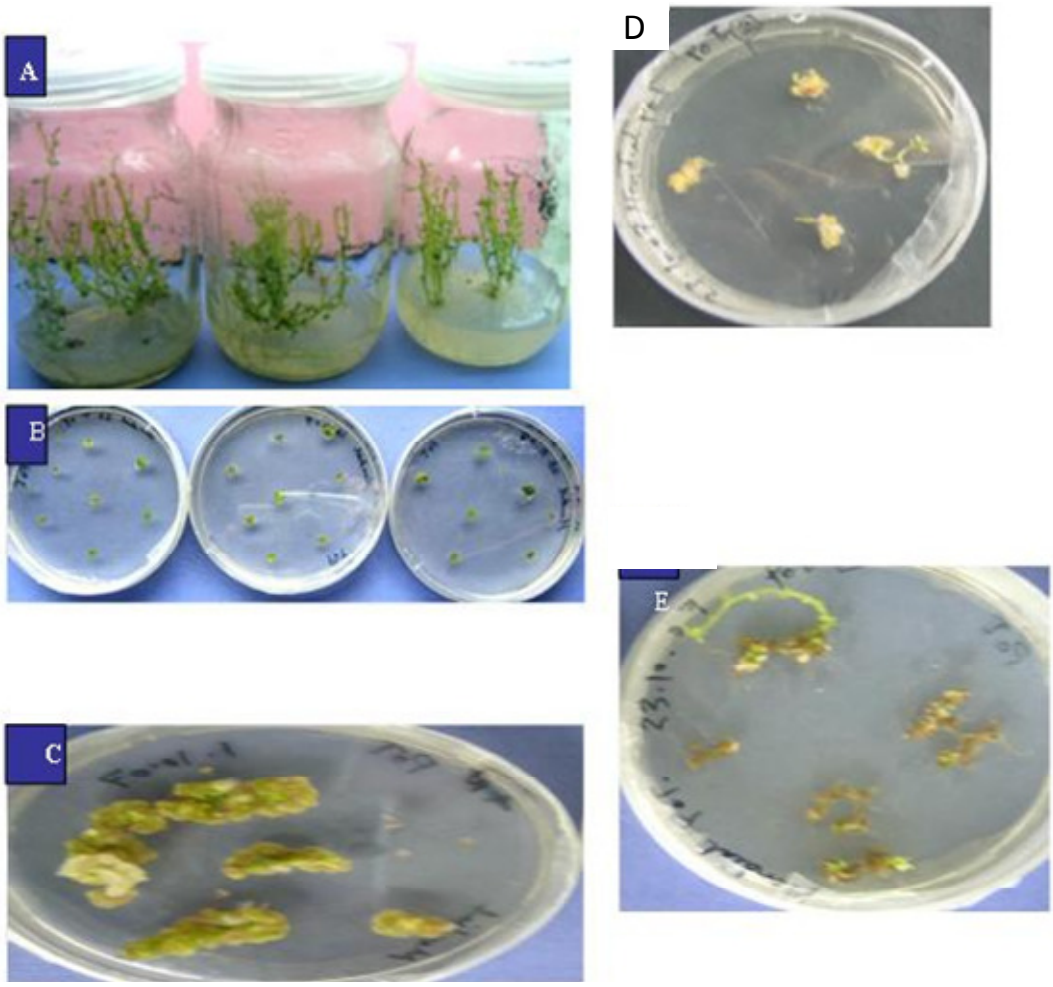

Figure 2. The formation process of regeneration of potato plants from leaf-derived callus. (A) Induction of callus around the leaf explants after 3 weeks of culturing on MS + 1.0 mg/l BA and 1.5 mg/l 2,4-D; (B) Proliferation of leaf base callus with green spots on the same callus initiation medium, MS + 1.0 mg/l BA and 1.5 mg/l 2,4-D after four weeks; (C) Green to purple-coloured shoots from the leaf base-derived callus of potato on transfer to MS + 1.5 mg/l BA and 1.0 mg/l NAA and 1.0 mM 2ip. (D) Regenerating callus with emergence of shoot meristemoids, after 8 weeks of culture. (E) Plantlets rooting from transfer of shoots on basal-MS medium. Potato plantlets adapted to free living conditions.

cry genes.

Sequence analysis

Results show that the fragment was 1.4 kp for isolated *cryIC* and 1.4 kp for *cryIC Stander* genes. GenScan and CLC analysis both predicted that the DNA fragment had two same active and AT rich repeat sides, containing a full-length open reading frame (ORF) of 1400 bp encoding 44 amino acids.

BLAST result showed that the predicted ORF had 99% identity to the published *B. thuringiensis cryIC* Stander gene *cryIC* (nucleotide sequence of cry IC gene

(Accession number: GenBank accession no. x96682.1), which is a member of the *B. thuringiensis cryIC* Stander gene, nucleotide binding, cry protein repeat family.

The potential open reading frame encoded for 44 amino acid residues and contained a potential repeat motif. The deduced amino acids sequence showed extensive homology with each other and the conserved active repeats (Figure 3). The detection of *cry* gene effective to insects in Order Lepidoptera was carried out using polymerase chain reaction (PCR). The PCR products were cloned, sequenced and subjected to BLAST search at NCBI. The full length *cry*1C were also cloned by PCR walking technique, sequenced and analyzed.

Digestion analysis

The fragment was cloned into the pSC-T vector with site-specific recombination. Recombinant plasmid pSC-T was confirmed by digestion of XbaI and BamHI.

PCR screening

PCR screening using different primers of pSC-T-cryIC construct (M13F and M13R) primers is present in the pSC-T- vector.

Expression of *cryICeg* gene and *cryICst* genes in pET29a

cryICeg and *cryICst* genes from isolated *cryIC* which is 1.4 kp long for *cryIC Stander* genes; 44 kDa protein, play a key role in the insecticidal activity and induces systemic resistance in susceptible plants against insect. The highly conserved regions among all *cry*1C genes were selected and used in primer design. The designed primers for PCR walking are listed in Table 2.

An extended multiplex PCR method was established to rapidly identify and classify *B. thuringiensis* strains containing cry (crystal protein) genes toxic to species of Lepidoptera, Coleoptera and Diptera. The technique enriches current strategies and simplifies the initial stages of large-scale screening of cry genes by pinpointing isolates that contain specific genes or unique combinations of interest with potential insecticidal activities, thus facilitating subsequent toxicity assays. Three pairs of universal primers were designed to probe the highly conserved sequences and classify them.

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). Several plants have been transformed by insecticidal protein coding genes isolated from *B. thuringiensis*. These plants exhibited varying degrees of protection against insect predation. It was hypothesized that prokaryotic gene may not be well expressed in plants and in part be responsible for observed limited protection in transgenic plants.

In addition, it was postulated that localized regions of A/T resembling plant introns and ATTTA sequences may destabilize mRNA of insecticidal protein coding genes leading only to partial protection against insect attack with low degree of protection against insect predation. Presently, it is not clear if these structural differences are solely responsible for the observed high level of expression and protection against insect predation.

***In vitro* regeneration of potato**

Transgenic plants have now become a commercial reality and genetic transformation is routinely achieved in most crop species. However, achieving a high expression of the introduced foreign gene in plant cells is still a challenging task, especially, in those projects aiming to develop transgenic crop plants that express insecticidal, antifungal, antibacterial and antiviral proteins. High level expression is equally important in those projects that aim at using transgenic plants as "biofactories" to produce novel enzymes, pharmaceuticals and industrial compounds. Therefore, it is critical that introduced genes express efficiently and produce the corresponding proteins in large amounts to make the venture economically feasible.

Callus induction and plant regeneration

Leaf explants initiated callus within 10 to 36 days when cultured on media containing different combinations of BAP, NAA and 2,4-D. Callus was characterized by globular structures, smooth and shiny creamy or green color. Repetitive callus grew from the initial leaf callus. Callus grew on medium containing MS media supplemented with 3 or 4% sucrose and MS + 1.5 mg/l BA and 1.0 mg/l NAA after four weeks.

Shoot formation

The comparisons of the effects of five different media on shoot regeneration from calluses of potato were conducted in this study. The result demonstrates that there was a significant correlation with BAP concentrations and the differentiation response. Higher concentration of (2.0 mg/l) BAP had no positive effect on shoot regeneration from calluses, while lower concentration of (0.5 mg/ l) BAP could reduce the rate of shoot regeneration from calluses. The optimum media for shoot regeneration from calluses was considered as MS

plus 1.5 mg/l BAP (Figure 2A). The results also indicated that the effects of differentiation might differ in calluses with different colors on optimum MS media of shoot regeneration from calluses with 1.0 mg/l BAP + 1.0 mg/l NAA. The rate of shoot regeneration from yellow calluses reached 80%; the rate of shoot regeneration from light yellow calluses was 24.0% after 2 subcultures, while the white sticky calluses could not generate shoots on the differentiation medium, and calluses continuously grew. When the concentration of BAP was 1.0 or 1.5 mg/l, the shoot grew faster as NAA concentration increased from 1.0 to 1.5 mg/l, but the speed of plant growth slowed down when NAA concentration was above 0.5 mg/l. The MS media with 1.0 mg/l BAP and 1.0 mg/l NAA could not only stimulate shoot growth, but also promoted formation and proliferation of small shoots (Figure 2B). The MS media with 1.5 mg/l BAP, 1.0 mg/l NAA and 1.0 mM 2ip were demonstrated to be the best media formulation for shoot growth.

Root formation

Rooting rate of shoots reached 95.0% on all rooting media, but there were marked differences in root qualities presented. According to root quantities and diameters, the optimum rooting medium was identified to be MS + 0.3 mg /l NAA. Roots began to generate on the 10th day. After 18 days, roots developed to 0.9 cm in length, 1.8 mm in diameter and six or seven in number (Figure 2C). Plantlets with roots were transplanted to the plastic pots, and then to the clay pots (Figure 2E). The survival rate of transplanting plantlets was 92.0%.

Figure 2 shows the protocols for the potato plant regeneration from leaf explants base-derived callus of potato starting from leaf explants and the established protocols from next figures as follows: The experiments indicated that NAA (1-naphthylacetic acid), BA and sucrose were the major factors influencing induction of somatic embryogenesis in potato. Less than 3% sucrose, induced friable callus regardless of the growth regulators combinations were used whereas 3 and 4% sucrose induced embryogenic callus. 2,4-D (0.5 or 1.5 mg/l) was the second major factor, followed by BA. The interaction between the sucrose and growth regulators significantly influenced somatic embryogenesis induction. Optimum induction was achieved with 3% sucrose, 1.0 mg/l 2,4-D and 0.1 (or 1.0 mg/l) BA after 4 weeks of culture.

Generation of stable transgenic potato plants

Agrobacterium mediated leaf disk method was used for transformation. Kanamycin at 100 to 200 mg/L was used to select a range of transgenic plants for GUS expression. A total of 50 independently transformed plants were selected and screened for the presence of GUS, cryIC sequences and the GUS expression. A frequency of 5 to 20 plantlets were obtained per explant, which was comparable to the control vector (pBI121) transformation frequency indicating that the cloning of cryIC gene had no adverse effects on the overall transformation frequencies. Transformed plants rooted normally and no abnormality associated with the transformation was observed. Transformed shoots rooted in the presence of 100 mg/L kanamycin and all the plants that were transferred to greenhouse grew normally and set seeds.

To evaluate the effect of NAA on shoot rooting, NAA was applied as a rooting medium. NAA had been demonstrated as the most suitable auxin for rooting in a number of plant species in several *in vitro* trails (Suzuki and Nakano, 2001; Maliti et al., 2005). However, the effects of different NAA concentrations on shoot rooting were not reported (Shuto et al., 2004; Ma et al., 2004). In this study, optimum medium MS + 0.6 mg NAA was demonstrated to have significantly better performance than that of non-plant growth regulator medium, regardless of root sizes and quantities.

Polymerase chain reaction

All colonies of the *cryIC–GUS* were subjected to a PCR with GUS gene as the universal and specific primers. PCR analysis was used to outline the products amplified from positive colonies. Three positive colonies were observed. The positive corresponding colonies were sent for sequencing. Cloning of the cryIC–GUS cassette was further confirmed by PCR for the plasmid DNA (*cryIC–GUS*) from both ends using the GUS5 and GUS3 primers present in the pBI121 vector.

All the constructs were introduced into *Agrobacterium tumefaciens* LB4404 by freeze-thaw method. *Agrobacterium*-mediated potato transformation was performed as described by Ooms et al. (1987) and Ottaviani et al. (1993). Potato was used for transformation with the vector pBI121 (sense gene). Regenerated transgenic plants were screened for *GUS* gene by PCR. After seven days of co-cultivation with *A. tumefaciens* strain harbouring the plasmid pBI121, a transient GUS assay indicated the transfer of the *gusA* gene to the plant cell. Following two months growth on selective medium, a second *GUS* assay was performed to test for the stable integration of the *gusA* gene in the plant genome. Majority (>85%) of the tested leaves stained blue and the results of these two assays, together with F0 cultivars on selective media, provided a strong indication of stable integration of the T-DNA in the host genome.

Our present results suggest that transgenic method is a feasible approach to improve the potato resistance. In addition, this method offered a new way to breed potato insect resistant varieties for other crops that can be

seriously damaged by insect and for which no genetic resources of resistance have been identified, like eggplant, cotton, cucumber and tomato, etc. A possible explanation for this was either protein encoded by this gene was unstable or the native promoter could not properly work, or some other factors involved in its pathway could not work properly. In this study, we replaced the native promoter of the gene with cauliflower mosaic virus (CaMV) 35S promoter, a stronger and constitutive promoter in order to figure out whether the instability is due to the gene expression or the activity of the promoter.

Production of transgenic tobacco plants

In this case, the efficient introduction of constructed plasmids into *A. tumefaciens* is of great practical importance. Transformation was done with the calcium chloride treatment with freeze-thaw prior to stable transformation, which is not suitable for the expression. Transformation frequencies of up to 10^7 transformants/□g DNA using electroporation have been reported. The transfer of naked DNA into plants by particle bombardment can be used as a rapid test for protein expression of large amounts of foreign proteins in plants. This technique has received far more attention for the regeneration of transgenic plants, in particular cereal crops. However, the obtained results refer to the successful transformation in tobacco plants.

In conclusion, an efficient plant regeneration system was established. The high rates of shoot regeneration from calluses, rooting and transplanting survival were achieved.

Results show that the transient gene expression in tobacco plants has advantages over the generation of stable transformed transgenic plants and it seems particularly suitable for verifying that the gene product is functional and stable before moving on to large-scale production in transgenic tobacco and potato. The results show that the tobacco leaf was transformed with plasmid DNA for transient assay of GUS. We can note the presence of blue spots on slices transformed with pBI21-CaMV 35S–cryIC-GUS, plasmid DNA. While no blue spots could be seen on the control leaf bombarded with pUC18 DNA as control plant. The generation of stably transformed transgenic plants requires a considerable investment in time before the expressed proteins can be analysed. In contrast, transient gene expression systems are fast, flexible and are not influenced by positional effects potentially bias by gene-expression levels (Kapila et al., 1996; Scholthof et al., 1996). Heterologous gene expression can be induced at an advanced developmental stage, which avoids any potential negative effects on the developing plant and can optimize the yield of the desired product.

Furthermore, using viral vectors for transient protein expression (Scholthof et al., 1996) has attracted commercial interest because it can rapidly produce high yields of protein on a field scale. 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). Several plants have been transformed by insecticidal protein coding genes isolated from *B. thuringiensis* (Strizhov et al., 1996). These plants exhibited varying degrees of protection against insect predation. It was hypothesized that prokaryotic gene may not be well expressed in plants and in part be responsible for observed limited protection in transgenic plants (Perlak et al., 1991; Tailor et al., 1992; Vaeck et al., 1987).

In addition, it was postulated that localized regions of A/T resembling plant introns and ATTTA sequences may destabilize mRNA of insecticidal protein coding genes leading only to partial protection against insect attack.

Presently, it is not clear if these structural differences are solely responsible for observed high level of expression and protection against insect predation. Nevertheless, it is apparent that sequence divergence of different insecticidal toxin proteins coding genes represents a gene pool which could be used for obtaining transgenic plants without resorting to drastic modifications in the sequence composition. We have demonstrated that the toxic domain of a wild-type *B. thuringiensis* (Bt) *cry1C* gene could be expressed efficiently in the potato and tobacco plants, resulting to high resistance for insects.

Abbreviations:

RFLP, Fragment length polymorphism; **NCBI,** Center for Biotechnology Information; **BLAST,** Basic Local Alignment Search Tool; **ORF,** open reading frame; **MS,** Murashige and Skoog; **NAA,** naphthalene acetic acid; **PCR,** polymerase chain reaction; **BA,** benzyladenine.

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