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Short Communication

Expression of a Modified *Cry1Ie* Gene in *E. coli* and in Transgenic Tobacco Confers Resistance to Corn Borer

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Abstract The wild-type *Cry1Ie* gene from *Bacillus thuringiensis* was modified for its efficient expression in transgenic plants. Modified *Cry1Ie* gene (designated as *Cry1Iem*) was cloned into prokaryotic expression vector pET28b and its expression in *E. coli* was confirmed by SDS-PAGE analysis. Bioassays using crude expression products in *E. coli* revealed that Cry1Iem protein had a similar toxicity to corn borer as wild-type Cry1Ie. *Cry1Iem* gene was then inserted downstream of the maize ubiquitin-1 promoter in plant expression vector p3301. Transgenic tobacco plants carrying *Cry1Iem* showed insecticidal activity against corn borer.

Key words *Bacillus thuringiensis*; *Cry1Iem*; transgenic tobacco; insect-resistance

Gene transfer technology provides an alternative approach to breed insect-resistant crops. Insect-resistant genes from plants or microbes could be introduced into plants and the expressed insecticidal protein in plant cells could kill the target insects. Transgenic plants expressing a corresponding insecticidal crystal protein gene from *Bacillus thuringiensis* (Bt) have been developed since the early 1980s [1,2]. Analysis of Bt gene sequences revealed that they contain numerous motifs seldom found in plant exons including: stretches of AT-rich sequences resembling plant mRNA processing signals [3]; plant polyadenylation signals, ATTTA sequences identified to be responsible for the destabilization of mRNA [4]; and codons which are rarely used in plant genes. To increase the expression of insecticidal proteins, synthetic Bt genes have been developed and used for transgenic plants [5–10]. Field trails of transgenic maize plants expressing synthetic Bt genes have been demonstrated to be toxic against corn borer [11].

The expressed insecticidal protein in most of the Bt transgenic plants currently marketed was either *Cry1A* (*Cry1Ac*/*Cry1Ab*) or *Cry9C* [12–14]. Several insect species have evolved resistance to Bt insecticidal proteins. During the application of Bt-transgenic crops, several resistance management strategies have been proposed, such as using new Bt gene, tissue- or developmental stage-specific expression of Bt genes, a combination of toxins with different modes of action and the use of refuges [15,16].

Here, we reported the modification of the nucleotide sequence of *Cry1Ie* gene, a novel *Cry1I*-type gene [17], and the expression of modified *Cry1Ie* gene (*Cry1Iem*) in *E. coli* and in transgenic tobacco to verify the toxicity of Cry1Iem protein to the main corn pest—Asian corn borer.

Materials and Methods

Materials

The restriction endonucleases, *Taq* DNA polymerase, IPTG, and T4 DNA ligase were obtained from Promega. The expression vector pET28b, *E. coli* host strain BL21 came from Novagen Co. Plasmid p3301 [18] and pAHC17 [19] were kept in our laboratory. All other chemicals were of analytical grade.

Modification of *Cry1Ie* gene

Design of the synthetic gene was based on the sequence

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of the corresponding wild type gene (GenBank accession No. AF211190). Modifications in the DNA sequence did not alter the amino acid sequence of the Cry1Ie protein. Codons were modified according to the preferential codon usage in plants [20]. Meanwhile, such DNA sequences as the potential polyadenylation signal, 4 or more consecutive adenine (A) or thymine (T) nucleotides [21], ATTTA sequence which may cause mRNA instability [22], and all motifs containing five or more G+C or A+T nucleotides were removed or modified. Sequences around the translation initiation site were also changed to conform to the eukaryotic consensus sequence [23]. Modified *Cry1Ie* gene was designated as *Cry1Iem*.

Construction of the expression plasmid

Cry1Iem gene was expressed in *E. coli* with the vector pET28b. The expression plasmid pETIe was constructed by ligating the 1.9 kb *Nhe*I-*Eco*RI fragment of *Cry1Iem* into pET28b. Sequencing and the restriction enzyme digestion confirmed that the ligation was successful.
Maize ubiquitin promoter was isolated from plasmid pAHC17 and ligated to the 5' end of *Cry1Iem* gene. Ubi-*Cry1Iem* *Hin*dIII/*Bst*EII fragment was inserted into the plant expression vectors p3301 to form a new plasmid pUbiIe, in which *Cry1Iem* located between the ubiquitin promoter and NOS terminator sequence.

Expression and purification of Cry1Iem protein in *E. coli*

E. coli BL21 competent cells were transformed with the expression plasmid pETIe. The transformants were grown at 37 °C in LB medium, 10 ml overnight culture of a single colony from the transformants was added into 1 L LB medium and cultured to A_{600} 0.4–0.6, then IPTG was added to a final concentration of 0.5 mM and the cells were incubated at the same condition for another 4 h. Cells were harvested by centrifugation at 4000 r/min for 30 min and resuspended in lysis buffer (2 mM Tris-HCl, 0.2 mM $CaCl_2$, pH 8.0), then disrupted by ultrasonication (60 times of 4 seconds pulses separated by 6 seconds of cooling). The inclusion bodies of Cry1Ie were washed with a wash buffer (2 M urea, 50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 8.0). Protein samples were analyzed by electrophoresis on 10% SDS-PAGE under denaturing conditions.

Transformation of tobacco

The plant expression plasmid pUbiIe was transferred into competent cells of *Agrobacterium tumefaciens* (strain LBA4404) by frozen-thaw treatment. The transformed *Agrobacterium* were selected on YEB-agar plates with 50 mg/L of kanamycin and 50 mg/L of streptomycin. Recombinant *Agrobacterium* were infiltrated into the young tobacco leaves according to Horsch *et al*. [24].

Southern blot analysis of regenerated tobacco plants

Genomic DNA was isolated from leaves of tobacco plant as previously described [25]. Each sample was digested with *Eco*RI/*Bst*EII, separated on 0.8% agarose gel, and transferred to nylon membranes. Probe for *Cry1Iem* gene was amplified by PCR and separated on 0.8% of agarose. A 1.3 kb fragment was purified from the gel and labeled with [α-^{32}P]dCTP. Standard procedures were used for Southern blot analysis [26].

Insect bioassay

Cry1Iem protein inclusions were purified from *E. coli* cells and concentrated according to the method of Shin *et al*. [27]. Effectiveness of Cry1Iem protein inclusion to the Asian corn borer was tested by dose-mortality response. The procedure of the assay was identical to that described for the transgenic plants except the liquid ingredient was used to substitute the plant materials. Cry1Ie protein was added to the liquid ingredients (distilled water) of the diet. Six to seven different concentrations of this toxin were used. The estimation of LC_{50}, slope and concentration-mortality line was fitted using probit analysis.

Resistance of transgenic tobacco plants were evaluated with fresh leaves using a diet-incorporated technique [28]. The agar-free semiartificial diet of Asian corn borer (*Ostrinia furnacalis*) was adapted for the bioassay. All solid ingredients of the diet were blended and finely ground with a kitchen grinder. Five grams of fresh tobacco leaves from transgenic plants were ground with 30 ml of the extraction buffer (50 mM Na_2CO_3, 100 mM NaCl, 0.05% Triton X-100, 0.05% Tween-20, 1 μM leupeptin, pH 9.5). After centrifugation, the supernatant was poured into a flask that contained 30 g of processed diet ingredients to form the testing medium. Bioassays were performed in 48-well trays in triplicate. Each well was filled with about 1.2 g of testing medium and infested with a neonate larva of Asian corn borer. All trays were moved to a rearing room at 70%–80% RH, 26–28 °C, and a photoperiod of 16:8 h (L:D). Survivors and larval weights were recorded after 7 d.

Results

Modification of *Cry1Ie*

Table 1 Sequence comparison of *Cry1Ie* with *Cry1Iem*

Gene	GC%	Potential poly(A) signals	ATTTA sequences	AT-rich regions	Modified codons	bps changed
Cry1Ie	37.04	2	8	0	–	–
Cry1Iem	47.22	0	0	0	298/648	350/1944

The purpose of redesigning *cry1Ie* was to create a synthetic gene that would be expressed highly in plant cells. The sequences of the wild-type *Cry1Ie* gene from nucleotide 1 to 1944 (amino acid 1 to 648) were partially modified. The wild *Cry1Ie* gene codons were replaced whenever possible by the plant bias codons without changing the amino acid composition, and the potential poly(A) signals AATAAA and AATAAT, the eukaryotic mRNA degradation signals and poly-ATTTA motifs existed in the *Cry1Ie* were eliminated (Table 1). The modified *Cry1Ie* gene was named as *Cry1Iem*.

Expression and purification of Cry1Iem in *E. coli*

The 1.9 kb synthetic *Cry1Iem* fragment was inserted into *Nhe*I-*Eco*RI sites of the pET28b vector, generating pETIe. The *E. coli* BL21 cells containing the expression plasmid pETIe produced the expected 74 kD fusion protein as revealed by SDS-PAGE analysis (Fig. 1). The expressed product formed inclusions in *E. coli*.

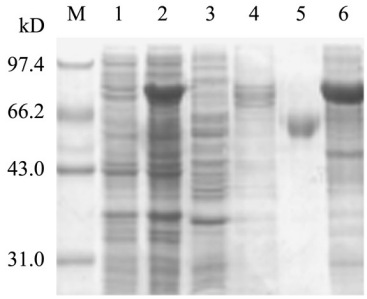

Fig. 1 Expression and purification of Cry1Iem in *E. coli*
M, protein marker; 1, transformed with pETIe, without IPTG induction; 2, transformed with pETIe, with IPTG induction; 3, transformed with plasmid pET28b; 4, supernatant from *E. coli* containing pETIe; 5, 1 μg BSA as control; 6, purified inclusion protein from *E. coli* containing pETIe.

Insect bioassays of Cry1Iem protein extracts from *E. coli*

The purified fusion protein Cry1Iem was tested for insecticidal activities against corn borer larvae. The results showed that Cry1Iem was active against the Asian corn borer. LC_{50} and LC_{90} were 6.58 μg/g (95% Fiducial Limits: 1.91–17.79) and 68.30 μg/g (95% Fiducial Limits: 23.93–692.73), respectively. The data indicated that the protein encoded by *Cry1Iem* gene retained the insecticidal activity against corn borer larvae as wild Cry1Ie.

Production and molecular characterization of transgenic tobacco plants

Binary vector pUbiIe (Fig. 2) were constructed and transformed into *A. tumefaciens* LBA4404, which were used for tobacco leaf disc transformation. After 6 weeks of culture, 20 tobacco plantlets were regenerated from *Agrobacterium*-infected leaf segments on regeneration medium containing 10 mg/L Basta. Among them, 14 plants gave an expected PCR product for *Cry1Iem* gene and their transgenic nature were further confirmed by Southern blot analysis.

*Eco*RI/*Bst*EII-digested genomic DNA extracted from selected tobacco plants were hybridized with α -^{32}P-labled 1.3 kb fragment of *Cry1Iem* gene. The results showed that 9 plants had a 2.5 kb band including part of ubiquitin promoter and *Cry1Iem* gene (Fig. 3), which indicated that *Cry1Iem* had integrated into tobacco genome.

Insect bioassays of transgenic plants

The 9 transgenic tobacco plants were tested for

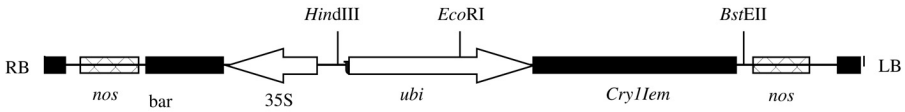

Fig. 2 Structure of T-DNA region of the binary vector pUbiIe
LB, left T-DNA border; RB, right T-DNA border; 35S, CaMV 35S promoter; bar, phosphinothricin acetyltransferase gene; *nos*, nos terminator; *ubi*, ubiquitin promoter; *Cry1Iem*, modified *Cry1Ie* gene.

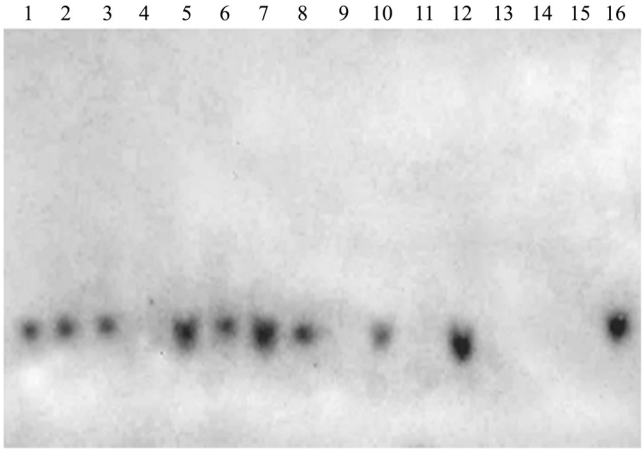

Fig. 3 Southern blot analysis of transgenic tobacco plants with *Cry1Iem*

1–14, DNA from PCR-positive tobacco plants with Cry1Iem; 15, DNA from non-transgenic tobacco plants; 16, plasmid pUbiIe as positive control.

resistance to corn borer larvae. Each sample was infested with 48 larvae and both survived and dead larvae were recorded after 7 days. The mortality and mean weight of tested larvae were shown in Fig. 4. The results showed that transgenic tobacco plants were toxic to Asian corn borer.

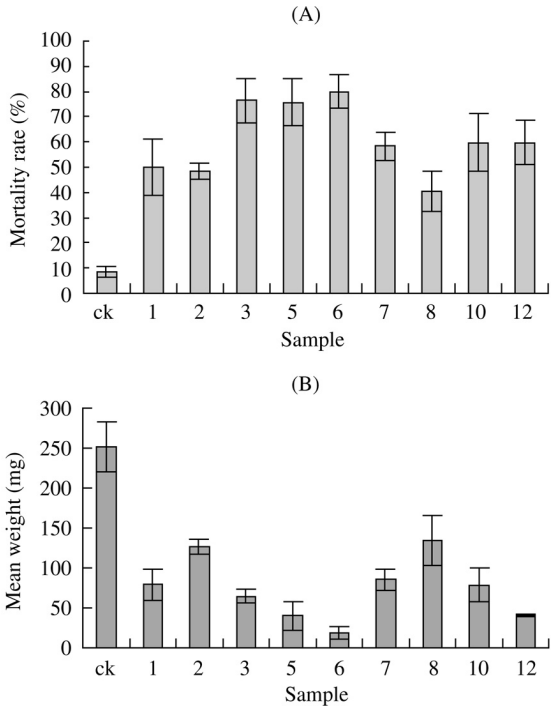

Fig. 4 Insect mortality (A) and average weight (B) of the tested larvae on transgenic tobacco plants

ck, non-transformed tobacco plants; sample numbers 1, 2, 3, 5, 6, 7, 8, 10 and 12 represent transgenic tobacco plants containing *Cry1Iem.*

Discussion

Many reports have demonstrated that the native *Bacillus thuringiensis* genes were very poorly expressed in transgenic plants and that modification of their nucleotide sequence significantly enhanced the expression level of these genes [5,7]. It has been suggested that the low expression of crystal protein genes in plants is a consequence of both poor transcription and translation [29,30]. Here, we reported that the wild type *Cry1Ie* gene was modified with the aim to its high expression in plant cells. The modification was based on eliminating potential poly(A) signals and using plant bias codons. Out of 648 codons in *Cry1Ie* coding region, 298 were modified. The G+C content of *Cry1Iem* gene was 47.22%, while that of wild *Cry1Ie* gene was 37.04%.

The synthetic *Cry1Iem* gene could express the expected 74 kD protein in *E. coli*. The results of insect assay showed that purified proteins were lethal to corn borer larvae. To confirm that the synthetic gene would function in transgenic tobacco plants, insect bioassays of transgenic tobacco leaves were carried out. The results showed that transgenic tobacco plants were toxic to corn borer larvae. Larvae feeding on transgenic tobacco plants showed significantly reduced growth rates and higher levels of larval mortality in comparison with that on control plants. Variability in larval mortality and growth rate reduction was observed among transgenic tobacco plants, which may be attributed to the difference in the expression level of *Cry1Iem* in these plants. Generally, transgenes were controlled with 35S promoter that is very strong in transgenic tobacco. In our study, *Cry1Iem* was controlled with ubiquitin promoter that is very strong in monocot plants and weaker than 35S promoter in transgenic tobacco. The insect resistance could be higher if *Cry1Iem* gene was controlled with 35S promoter in transgenic tobacco plants.

It has been reported that several insect pests have developed resistance against Bt proteins [31–33]. Therefore, isolation of different new Bt gene(s) having different mode of action against target insects was crucial for solving this problem. Song *et al.* [17] have reported that Cry1Ie protein was highly toxic to Asian corn borer, the diamondback moth and soybean pod borer. In this report, modified *Cry1Iem* has been expressed in *E. coli* and transgenic tobacco plants, and the Cry1Iem protein thus produced was highly toxic to the larvae of Asian corn borer.

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