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Signal Peptide of Potato PinII Enhances the Expression of *Cry1Ac* in Transgenic Tobacco

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Abstract The modified Cry1Ac was expressed in transgenic tobacco plants. To allow secretion of the Cry1Ac protein into the intercellular space, the signal peptide sequence of potato proteinase inhibitor II (pinII) was N-terminally fused to the Cry1Ac encoding region. Expression of Cry1Ac in transgenic tobacco plants was assayed with ELISA. The results showed that pinII signal peptide sequence enhanced the expression of Cry1Ac protein and led to the secretion of the Cry1Ac protein in transgenic tobacco plants. GFP gene was also fused to the signal peptide sequence and transformed to tobacco. The results of fluorescent detection showed that GFP had localized in the apoplast of transgenic plants.

Key words Cry1Ac; signal peptide; GFP; transgenic tobacco

Insect-resistant plants have been developed through expression of insecticidal proteins from *Bacillus thuringiensis* (Bt) in the early 1980s [1,2]. However, for control of insect pests, it is necessary to increase the expression of Bt protein overall or in specific plant tissues. To increase the expression level, synthetic Bt genes have been developed and used to produce transgenic plants [2–5].

A number of approaches have been taken to increase the expression level of foreign proteins in transgenic plants. Signal peptides, which could transport and locate polypeptides into specific organelles, were used to increase the expression of foreign genes [6,7]. The signal peptide of potato proteinase inhibitor II (pinII) could target the recombinant protein to the apoplast of transgenic plants [8–10]. Herbers *et al.* [8] reported that 37 kD *Clostridium thermocellum* xylanase fused to pinII signal peptide was synthesized in high level and correctly targeted to intercellular space. Biotin-binding proteins (avidin and streptavidin) were expressed in tobacco tissues by using N-terminal peptide of potato proteinase inhibitor, and the highest level observed reached 1.5% of leaf totalprotein [9]. Human serum albumin was also expressed by using pinII signal peptide, and successfully targeted to the apoplast as shown by subcellular localization [10]. However, it is an interesting question whether the signal peptide sequence could increase the expression of Bt insecticidal protein gene in transgenic plants.

In this work, the modified *cry1Ac* gene from Bt was expressed in transgenic tobacco plants with and without pinII signal peptide, under the control of maize ubiquitin promoter. It was also observed that green fluorescent protein (GFP) fused to pinII signal peptide was targeted to apoplast in transgenic tobacco plants.

Materials and Methods

Materials

Bacterial strains (*Escherichia coli* DH5α, *Agrobacterium tumefaciens* LBA4404), plasmids pAHC17 [11] and p3301 [12] were preserved in our laboratory. The plasmid pET30-actin-gfp [13] was kindly provided by Dr. Guoqin LIU, China Agricultural University. The restriction endonuleases, *Taq* DNA polymerase and T4 DNA ligase were obtained from Promega Company.

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Amplification of pinII signal peptide sequence and *gfp* gene

The primers for *pinII* signal peptide sequence were designed according to the reported *pinII* sequence [14]. Forward primer was 5′-GGATCCACAGAC-ACTCTTCACCCCAA-3′, and reverse primer 5′-CGGCCGGCAAGCCTTCGCATCAAC-3′. PCR amplification was carried out using the following program: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min; and finally, 72 °C for 10 min.

The PCR primers for *gfp* gene [13] were designed as follows: forward primer was 5′-CGGCCGATG-AGTAAAGGAGAAGAAC-3′ and the reverse primer was 5′-GGTCACCTTATTTGTATAGTTCATCCA-3′. PCR amplification was carried out using the following program: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min; and finally, 72 °C for 10 min.

Modification of *cry1Ac* gene

Design of the synthetic *cry1Ac* gene was based on the sequence of the corresponding wild type gene (GenBank accession No. AF148644). Modifications in the DNA sequence did not alter the amino acid sequence of the Cry1Ac protein. Codons were modified according to the preferential codon usage in plants [15]. The potential polyadenylation signal, four or more consecutive adenine (A) or thymine (T) nucleotides [16], ATTTA sequence [17], and all motifs containing five or more G+C or A+T nucleotides were removed. Sequences around the translation initiation site were changed to conform to the eukaryotic consensus sequence [18].

Construction of expression plasmids

Synthesized 1.9 kb *cry1Ac* fragment was ligated to pUC18 to generate plasmid pUC18Ac. The ubiquitin promoter fragment (2.0 kb) was isolated by *Hin*dIII/*Bam*HI digestion from pAHC17, and ligated to the pUC18Ac digested by the same enzymes to construct pUC18ubiAc. The pUC18ubiAc was digested with *Hin*dIII and *Bst*EII, and the fragment containing ubiquitin gene promoter and *cry1Ac* gene was inserted into p3301 digested with the same enzymes to construct plasmid p3301ubiAc. The signal peptide fragment of *pinII* digested with *Eco*52I and *Bam*HI was inserted between ubiquitin promoter and *Cry1Ac* in p3301ubiAc to construct the plasmid p3301ubisigAc. The *Eco*52I-*Bst*EII *Cry1Ac* fragment in p3301ubisigAc was replaced with *Eco*52I-*Bst*EII *gfp* fragment to construct the plasmid p3301ubisigGFP.

Transformation of tobacco

The plant expression plasmid p3301ubiAc, p3301-ubisigAc and p3301ubisigGFP were transferred into competent cells of *A. tumefaciens* strain LBA4404 by frozen-thaw treatment. The transformed *Agrobacterium* clonies 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.* [19].

PCR analysis of transgenic tobacco plants

Genomic DNA for PCR analysis was isolated from leaf tissues of tobacco plants as previously described [20]. PCR analysis was carried out according to Sambrook *et al.* [21].

Fluorescence spectrometry

A *Leica* fluorescence microscope was used to observe the location of GFP in transgenic tobacco leaves and images were photographed with cooled CCD (Micro MAX Princeton Instruments, Inc.).

Isolation of intercellular fluid

Isolation of intercellular fluid of tobacco leaves was carried out according to Börnke *et al.* [6]. After removal of the midrib, leaves were cut into pieces and subsequently infiltrated with 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) under vacuum for 10 min. Excess buffer was removed and the intercellular fluid was collected by means of centrifugation for 5 min at 4000 *g* and 4 °C.

ELISA and Western blot analysis of transgenic tobacco plants

Fresh tissues were collected from plants growing in the field or the greenhouse, and ground in 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). The protein extraction and ELISA were performed as described [22]. 10 mg/ml purified Cry1Ac protein from *E. coli* was diluted into various concentrations and used to plot the relationship between Cry1Ac content and the ELISA data (Fig. 1). The total protein content was determined according to Bradford [23]. Western blot analysis was performed according to the standard method [21]. The first antibody was the rabbit antiserum of Cry1Ac (1:2500) and the second antibody was the goat anti-rabbit serum conjugated with alkaline phosphatase (1:7500).

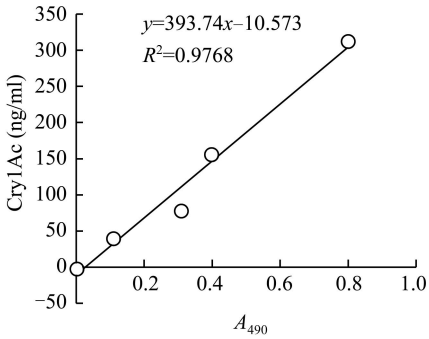


Fig. 1 The relationship between Cry1Ac concentration and ELISA data (A_{490})

Results

Amplification of DNA fragment encoding for pinII signal peptide

A fragment of 279 bp encoding pinII signal peptide was amplified by PCR and cloned into pGEM®-T easy vector. The amplified fragment was sequenced and confirmed by comparison with the reported sequence (data not shown).

Modification of *cry1Ac* gene

The sequence of the wild type *cry1Ac* from nucleotide 1 to 1854 (amino acid 1–618) was partially modified. The codons in the redesigned *cry1Ac* gene were replaced whenever possible by the plant bias codons without changing the amino-acid composition. At the same time, potential poly(A) signals and eukaryotic mRNA degradation signals in the synthetic *cry1Ac* gene were also eliminated (Table 1).

Plasmid construction

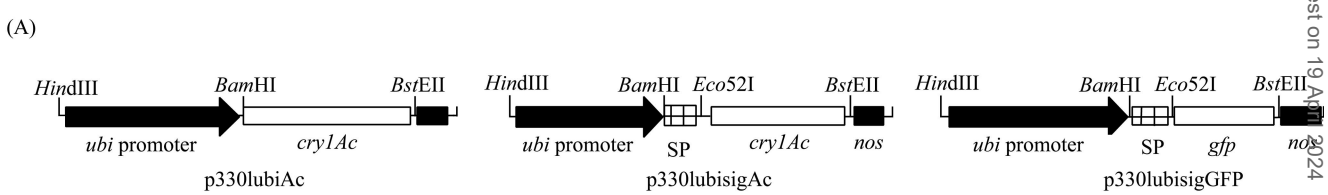
Plasmid p3301ubiAc was constructed in which *cry1Ac* gene was fused to ubiquitin promoter. The pinII signal peptide sequence was inserted between the ubiquitin promoter and *cry1Ac* without frame-shift to construct p3301ubisigAc. In p3301ubisigGFP, the *gfp* gene was fused behind the signal peptide in order to confirm the gene expression at the cellular level (Fig. 2).

Molecular analysis of transgenic tobacco plants

PCR amplification of signal peptide sequence, *cry1Ac*,

Table 1 Comparison of modified *cry1Ac* gene with wild type gene

Gene	GC%	Potential poly(A) signals	ATTTA sequences	AT-rich regions	Modified codons	Base pairs changed (bp)
Wild *cry1Ac*	37.3	3	10	1	–	–
Modified *cry1Ac*	64.8	0	0	0	495/618	624/1854



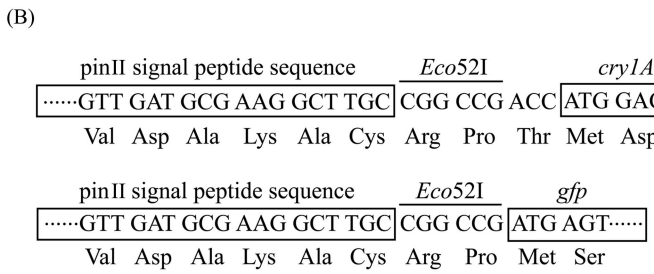


Fig. 2 Structure of plant expression vector p3301ubiAc, p3301ubisigAc and p3301ubisigGFP

(A) The schematic structure. (B) The fusion region between the pinII signal peptide and *cry1Ac*, *gfp* gene in more detail. In both construct, the pinII signal peptide derived N-terminal region ended three amino acids after the signal peptide cleavage site.

gfp was carried out. Specific 0.3 kb fragment of signal peptide sequence, 1.7 kb fragment of *cry1Ac* and 0.7 kb fragment of *gfp* were obtained from transgenic tobacco plants (Fig. 3). These results showed that *cry1Ac* and *gfp* gene have integrated into tobacco genome.

Fluorescent detection of *gfp*-transgenic tobacco plants

To target GFP into the intercellular space of plant cells, the potato pinII signal peptide was fused in front of *gfp* gene to construct the plasmid p3301ubisigGFP. Leaf trichomes of transgenic tobacco plants were used for fluorescent detection. Faint GFP fluorescence appeared in the apoplast of plant cells (Fig. 4), which indicated that the signal peptide targeted GFP into the intercellular space in transgenic tobacco plants.

Expression of Cry1Ac in transgenic tobacco plants

Transgenic tobacco plants, transformed respectively with p3301ubiAc and p3301ubisigAc, were chosen for Western blot analysis. The results showed that Cry1Ac protein expressed in transgenic tobacco was about 68 kD in molecular weight, which is consistent with the prediction according to the modified *cry1Ac* gene. Cry1Ac protein about 68 kD in molecular weight was also detected in intercellular fluid [Fig. 5(B), lane 2], which confirmed that the signal peptide targeted Cry1Ac protein into the intercellular space of transgenic plant cells, and the pinII signal peptide had been cleaved off when Cry1Ac protein transferred to the apoplast of transgenic plants.

ELISA analysis was carried out to quantify the expression of Cry1Ac protein in four transgenic tobacco lines, two of the lines carried *cry1Ac* with signal peptide (S7 and S14) and the other two lines carried *cry1Ac* without signal peptide (A3 and A6). Ten T_1 plants were measured in each line. The results showed that Cry1Ac protein content in transgenic tobacco lines S7 and S14 was significantly higher than that in lines A3 and A6 (Table 2), which indicated that the signal peptide of PinII from potato obviously increased the expression of Cry1Ac protein in transgenic tobacco plants.

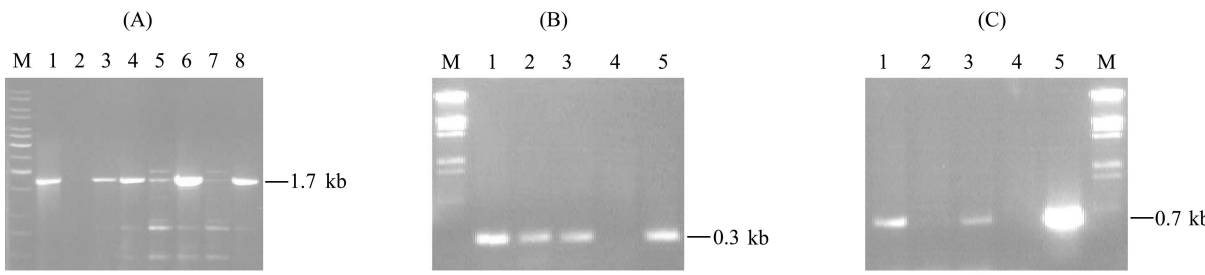


Fig. 3 PCR amplification of *cry1Ac*, signal peptide sequence and *gfp* gene

(A) *cry1Ac*. M, 1 kb DNA ladder; 1, plasmid p3301ubiAc as positive control; 2, untransgenic tobacco plants; 3–8, transgenic tobacco. (B) signal peptide sequence. (C) *gfp* gene. M, DNA marker (*Hin*dIII/*Eco*RI); 1–3, transgenic tobacco plants; 4, untransgenic tobacco plants; 5, plasmid p3301ubisigGFP as positive control.

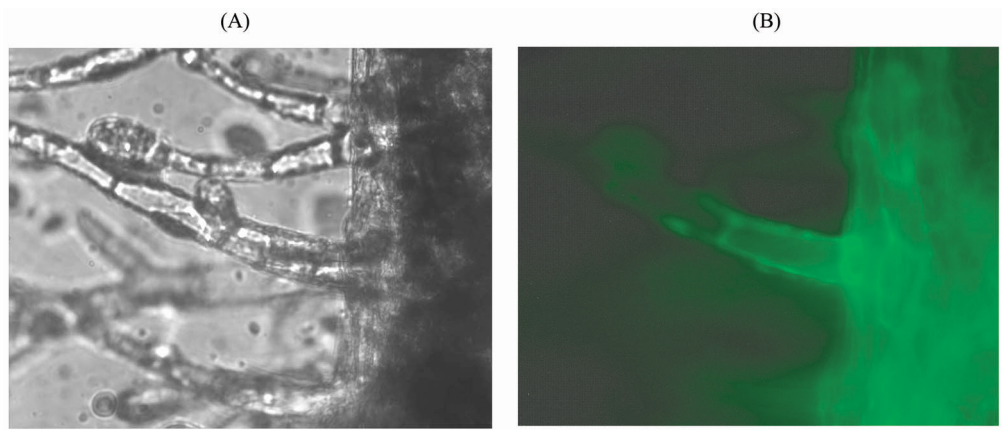


Fig. 4 Detection of green fluorescence in leaf epidermis trichome

(A) Under visible light. (B) Under green light.

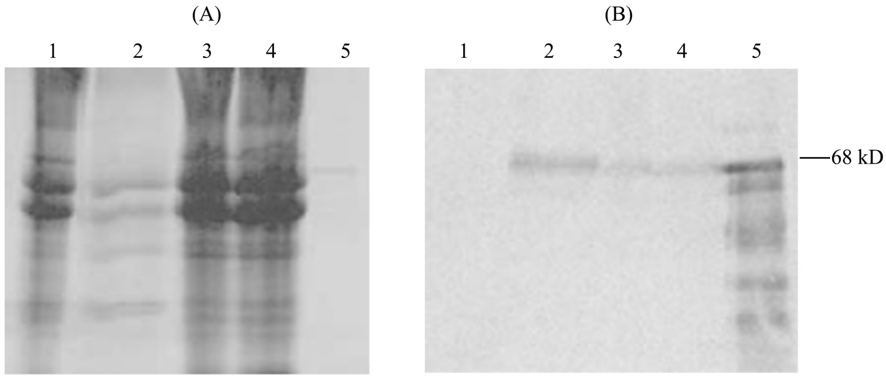


Fig. 5 Western blot analysis of transgenic tobacco plants

(A) SDS-PAGE. (B) Western blot. 1, untransgenic tobacco; 2, intercellular fluid from p3301ubisigAc transgenic tobacco; 3, total protein extracts from p3301ubisigAc transgenic tobacco; 4, total protein extracts from transgenic tobacco containing p3301ubiAc; 5, Cry1Ac protein extracts from *E. coli* as positive control.

Discussion

Many studies have demonstrated that 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 [5,24]. It has been suggested that the low expression of crystal protein genes in plants is a consequence of both poor transcription and translation [24,25]. Here, we reported that the wild type *cry1Ac* gene was modified with the aim to enhance its expression in plant cells. The modification was based on the elimination of potential poly(A) signals, the eukaryotic mRNA degradation signals and poly-ATTTA. In the modified *cry1Ac*, 495 codons were modified, which accounted for 80.1% of the gene. The G+C content of the modified *cry1Ac* gene was increased to 64.8%, while that of wild type *cry1Ac* was only 37.3%.

Signal peptide sequences transport and locate polypeptides to various organelles of cells. Many reports showed that some signal peptides changed the localization style of foreign proteins and also increased the expression of the foreign proteins in transgenic plants [6,7]. The signal peptide sequence of potato proteinase inhibitor II has been reported to increase the expression level of foreign protein by targeting proteins to the apoplasts in several transgenic plants [8–10]. To confirm whether pinII signal peptide could target the foreign protein to the apoplasts, *gfp* was fused behind the signal peptide sequence and transformed to tobacco. The results of fluorescent detection showed GFP localized in the apoplasts in transgenic tobacco plants. To achieve high expression of Cry1Ac protein in transgenic tobacco plants, we fused pinII signal peptide in front of *cry1Ac* gene. Western blot results showed the existence of Cry1Ac protein in the intercellular fluid of transgenic tobacco plants.

ELISA analysis showed the expression level of Cry1Ac protein in transgenic tobacco plants was very high with the highest level in leaf up to 0.366% of total protein. In our study, *cry1Ac* was controlled with maize ubiquitin promoter that has lower activity than 35S promoter in transgenic tobacco. Expression level of Cry1Ac would be higher if *cry1Ac* gene was controlled with 35S promoter. The expression level of Cry1Ac protein in transgenic tobacco plants with signal peptide of pinII was significantly higher than that without signal peptide. The plasmids p3301ubisigAc and p3301ubiAc have also been used to transform maize inbred lines, and the transgenic maize plants are being evaluated for their resistance to Asian corn borer.

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