Construction of a new plant expression vector containing two insect resistant genes and its expression in transgenic tobacco plants*

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Abstract A new plant expression vector (pBS29K-BA) containing two insect resistant genes, a synthetic chimeric gene *BtS29K* encoding the activated insecticidal protein Cry1Ac and a gene *API-BA* encoding the arrowhead (*Sagittaria sagittifolia* L.) proteinase inhibitor (API) A and B, is constructed. Transgenic tobacco plants expressing these two genes are obtained through Agrobacterium-mediated transformation of tobacco leaf discs. The average expression levels of Cry1Ac and API-BA proteins in transgenic plants are of 3.2 μg and 4.9 μg per gram fresh leaf respectively. The results of insecticidal assay of transgenic plants indicate that the pBS29K-BA transformed plants are more resistant to insect damage than the plants expressing the *Cry1Ac* gene or *API-BA* gene alone.

Keywords: *cry1Ac*, **arrowhead proteinase inhibitor, insect-resistant transgenic plants.**

Structural studies of crystal proteins from *Bacillus thurigiensis* (Bt) have suggested that the insecticidal activity results from the activiation of the Bt crystal protein by solubilization of the crystal proprotein (or called protoxin) in the alkaline gut of lepdopterian insects and by subsequent removal of both C- and N-terminals of the proprotein to yield the 65~70 kD activated toxin[1,2]. However the Bt toxin expressed in transgenic plants so far is the 3'-end truncated protoxin and still need to be activated by insect proteinases. If such protoxin is co-expressed in the plant with a proteinase inhibitor, the activation of the former in the insect gut will possibly be inhibited or interfered with by the latter. This might be one of the reasons for the lower insect resistance of transgenic plants that co-transformed with a *Bt* toxin gene and a proteinase inhibitor gene than those transformed with *Bt* gene alone[3].

To increase the expression level of *Bt* toxin gene and to stabilize the Bt toxin protein in transgenic plants, we synthesized a chimeric *Bt* toxin gene *BtS29K* which is composed of the DNA fragment encoding the activated protein of Cry1Ac and a signal peptide sequence at its 5'-end and a polypeptide KDEL coding sequence linked to its 3'-end. This chimeric *Bt* gene was further constructed into a plant expression vector that already contained the *API-BA* gene to form a recombinant plasmid, pBS29K-BA. In this paper, we report the construction of this expression vector and the molecular characterization of the transgenic plants that express Bt and API proteins.

1 Materials and methods

1.1 Bacterial strains and plasmids

Escherichia coli DH5 α, *Agrobacterium tumefaciens* LBA4404, plasmids pUC19, pD12[4], pBluescriptII SK^+ and pBin438[5] were preserved or constructed in our laboratory.

1.2 Synthesis of chimeric *cry1Ac* gene *BtS29K* and construction of the plant expression vector

Based on the DNA sequences of *Cry1Ac* genes and their deduced amino acid sequences from *Bacillus thuringiensis kurstak* HD-1 and HD-73[6,7], the plant codon usage[8] and nucleotide motifs affecting the stability and translation efficiency of eukaryotic mRNAs, a DNA fragment of 1755 bp encoding amino acid residues of 29~613 of the fully activated Cry1Ac protein was chemically synthesized[9] and designated *Btf*29.

To target the Cry1Ac protein into the endoplasmic reticulum (ER) in transgenic plants, a DNA sequence encoding the ER retaining polypeptide KDEL[10~12] was added to the 3'-end of *Btf*29 gene by PCR using a primer PV-9K (5' CACTC GAG

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ATC TCA AAG CTC GTC CTT TTC GAG TGT TGC AGT AAC TGG AAT GAA CTC AAA TCT GTC TAT GAT CAC ACC TGC) complementary to the 3'-end of *Btf*29 and cloned into *Bam*H1 and *Xho*I sites of pBluescript II SK^+ to form the recombinant plasmid pBt29K. To enable the translocation of the chimeric Bt protein, a DNA fragment encoding the murine κ light-chain signal peptide was added at the 5' end of *Bt29K* gene in pBt29K to form the recombinant plasmid pS29K. The chimeric *BtS29K* gene synthesized above was isolated from pS29K as a *Bam*HI and *Xho*I fragment and inserted into the *Bam*HI and *Sal*I sites of the binary vector pBin438[5] to form the plant expression vector pBS29K. This expression vector was transformed into *Agrobacterium tumefaciens* as described by An et al.[13].

1.3 Construction of the vector for expressing arrowhead proteinase inhibitor

1.3.1 Construction of a fusion protein gene of arrowhead proteinase inhibitor (API) B and A Two pairs of primers for *API-A* and *API-B* genes were used to amplify these two genes respectively. *API-B* primers were B_1(5' GCT GGA TCC ACC ATG GCG GCC TCC AAC GCT) and B_2(5' TGC CTG CAG AGA TCT CAT TGC GAG TGC GTC GAA). *API-A* primers were A_1(5' GTC GGA TCC TGC CAC GGA GAT CCC GTC) and A_2(5' TGC AAG CTT CTC GAG CTA CT G CGG TGC AGT TTT C). DNA fragments of *API-A* and *B* genes were amplified by PCR[14] using *API-A* and *API-B* cDNA clones in M13 phage provided by Professors Qi Zhengwu and Gong Zhenzhen, Institute of Biochemistry, Chinese Academy of Sciences, and were cloned into pUC19 to form pAHA and pAHB respectively. Fusion *API-BA* gene (pAHBA) was obtained by double-digestion of the *API-A* and *API-B* fragment with *Bam*H I-*Hin*d III and *Bam*H I-*Bgl* II and ligation.

1.3.2 Construction of the vector for expressing *API-BA* gene Fusion protein *API-BA* gene was recovered from pAH-BA as a *Bam*H I and *Xho*I fragment and cloned into pBin438[5] at *Bam*H I and *Sal*I sites to form the plant expression vector pBBA.

1.4 Construction of plant expression vector containing *BtS29K* and *API-BA* genes

The *Bam*H I-*Xho*I fragment of *API-BA* described above was inserted in *Bam*H I~*Sal*I sites of pD12[4] to form recombinant plasmid pDBA. The *API-BA* gene expression cassette was isolated from pDBA as a *Hin*d III fragment and inserted into *Hin*d III site of pBS29K to form the plant expression vector pBS29K-BA.

1.5 Transformation of tobacco and analysis of transgenic plants

Transformation of tobacco (*Nicotiana tabacum*) NC89 leaf tissues was carried out by *Agrobacterium*-mediated method[15]. Insect bioassay of transgenic tobacco plants was conducted as described in Ref. [5]. Tobacco DNA used for PCR detection was isolated according to Li et al.[5]. Gene specific primers for *Cry1Ac* amplification were $PmII^{(+)}$ (5'ATC TAT GCA GAG TCT TTC AGA) and $PLV\text{-}6^{(-)}$(5'GAG GTT ATC CAA GGA GGT). A fragment of 1370 bp should be amplified by using these two primers. For the amplification of *API-BA* gene, the primers are B_1 and A_2 as described in 1.3.1. A 1.2 kb PCR product was expected using this pair of primers.

For Southern blot analysis of transgenic plants, tobacco genomic DNA was isolated as described in Ref. [16]. The *Eco*R V-*Xho*I fragment of *BtS29K* gene or *Bam*H I-*Xho*I fragment of *API-BA* gene were ^{32}P-labeled and used as probes in Southern blot analysis.

1.6 Expression of two insecticidal proteins in transgenic plants

Fresh tobacco leaf samples of 100 mg were ground to powder in liquid nitrogen and then suspended in 100 μL 2 × sample buffer. The suspension was used for SDS-PAGE and Western blot analysis following the procedure in Ref. [14].

The inhibitory activity of API-BA against trypsin was determined as described by Hummel[17]. Protein concentration was determined by Bradford method[18].

1.7 Genetic analysis of transgenic plants

Segregation of kanamycin resistance in T_1 progeny of transgenic plants was checked as described in Ref. [5].

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2 Results and discussions

2.1 Synthesis of chimeric *Cry1Ac* gene *BtS29K* and construction of its plant expression vector

In order to increase the stability and accumulation of the Cry1Ac protein produced in transgenic plants, a chimeric *Bt* gene, *BtS29K*, composed of murine κ light-chain signal peptide-Btf29-KDEL coding sequence was synthesized based on the design reported by Schouten et al.[12]. In this chimeric gene, the sequence encoding the fully activated Cry1Ac protein is 1755 bp long. There are 342 bases changed, which will change 311 codons for amino acids. The changed codons account for 53.2% of the total codon numbers. The GC content in *Cry1Ac* gene is increased from 37% of the wild type gene to 47.4% of the synthesized gene, similar to a normal plant gene[9]. The synthetic *BtS29K* gene has a length of 1857 bp including the 1755 bp for the activated Cry1Ac, 72 bp for the signal peptide, 12bp at the 3' end for the KDEL polypeptide and a 15 bp spacer. This *BtS29K* gene fragment was inserted into pBin438 to form the plant expression vector pBS29K as shown in Fig. 1(a).

2.2 Construction of the plant expression vector pBS29K-BA containing the two insect-resistant genes

API-A and API-B have inhibitory activities against different types of proteinase[19]. To explore the inhibitory effect of API-A and API-B on insect, a fusion proteinase inhibitor gene *API-BA* was constructed, and structure of the plant expression vector pBBA for *API-BA* gene is shown in Fig. 1(b). A plant expression vector, pBS29K-BA, containing expression cassettes of both *BtS29K* and *API-BA* genes is shown in Fig. 1(c).

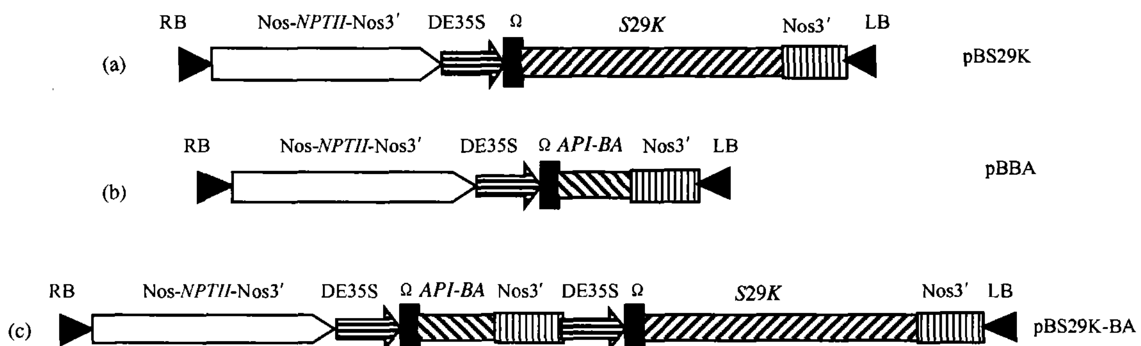

Fig. 1. Gene structure of three plant expression vectors.

2.3 PCR analysis and insecticidal assay of transgenic plants

About 150 kanamycin-resistant tobacco plants were regenerated from *Agrobacterium*-mediated transformation of leaf explants of tobacco NC89. The results of PCR analysis revealed that more than 90% of the regenerated plants could produce the expected gene-specific PCR product, implying that they are possibly transgenic. Electrophoresis patterns of the PCR products of some tobacco plants are shown in Fig. 2(a) and (b).

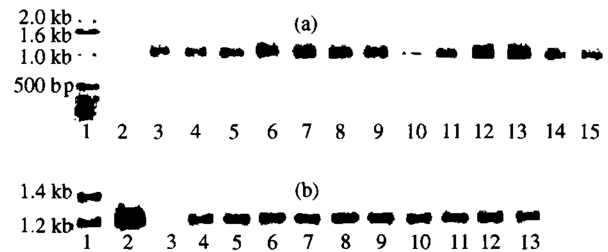

Fig. 2. PCR detection of some pBS29K-BA transformed tobacco plants.
(a) PCR products amplified by *Bt* gene specific primers. Lane 1, 1 kb DNA ladder; lane 2, control from non-transformed tobacoo; lanes 3~15, pBS29K-BA transformed plants. (b) PCR products amplified by *API-BA* gene-specific primers. Lane 1, λ-Ecot14I molecular marker; lane 2, pBS29KBA DNA as a positive control; lane 3, non-transfomed tobacco DNA; lanes 4~13, pBS29K-BA transformed plants.

The results of insect bioassay of PCR positive tobacco plants are summarized in Fig. 3, showing that the percentages of transgenic plants with medium to high insect resistance (mortality of H. *armigera* reached 70%~100%) are 54% and 39% of the total plants tested among *BtS29K* and *API-BA* transgenic

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plants respectively, while 64% could be obtained among pBS29K-BA transformed plants. The percentage of the plants with medium to high resistance transformed with the two insect resistant genes is significantly higher than those transformed with only one of the two genes, 10% higer than *BtS29K* plants and 25% higher than *API-BA* transformed plants. These results indicate that the chimeric *BtS29K* gene encoding fully activated Cry1Ac protein is favorable for construction of the expression vector with proteinase inhibitor genes and suggest that using the fully activated Cry1Ac and API-BA could lead to a higher insecticidal activity and wider spectrum of insect resistance to delay the development of insect tolerance against insect-resistant transgenic plants.

To evaluate the effect of *API-BA* gene on insect-resistance, besides recording the mortality of *API-BA* transgenic plants against cotton ballworm, the inhibitory effect on the larvae growth was also determined. The average weight of survived larvae grown on the leaves of transgenic plants at 5 dpi was only half of that grown on nontransformed plants (Fig.4). Results shown in Figs. 3 and 4 indicate that the fusion protein API-BA expressed in transgenic plants not only have insecticidal activity, but also have significant inhibitory effect on the growth of the tested insects. These results also indicate that *API-BA* gene could express normally in transgenic plants and confer insect-resistance on the plants.

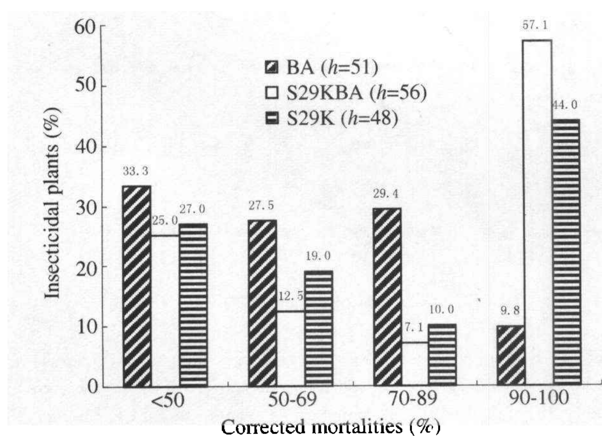

Fig. 3. Distribution of insect-resistance among transgenic tobacco plants. The numbers in parentheses behind the gene structure symbols represent the total plant number assayed. The corrected mortality is from the results at 5 dpi. The average motality of 10 non-transformed plants is 5.2%.

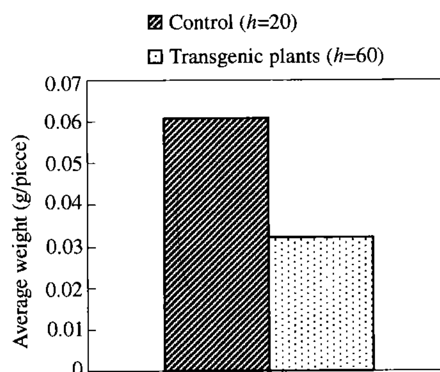

Fig. 4. Inhibition of cotton ballworm larvae growth on *API-BA* transgenic plants. Numbers in the parentheses represent the plant number used in the assay.

2.4 Southern blot analysis

Some PCR positive and insect-resistant plants were selected for Southern blot hybridization to confirm their transgenic nature. Fig. 5 shows the results of the transgenic plant DNA hybridized with ^{32}P-dCTP labeled *BtS29K* or *AIP-BA* gene probes respectively. It demonstrates that the plants analyzed are transgenic for both genes.

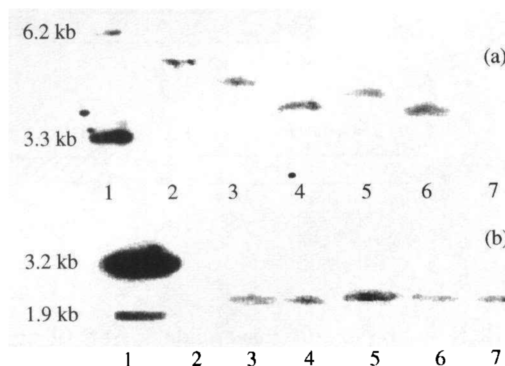

Fig. 5. Southern blot hybridization patterns of pBS29K-BA-transformed tobacco plants. (a) Hybridized with *Bt* gene probe. Lane 1, fragments (6.2 kb and 3.3 kb) containing *Bt* gene; lanes 2 ~6, *BtS29K-BA* transgenic plants; lane 7, non-transformed tobacco plant DNA; (b) hybridized with *API-BA* gene probe. Lane 1, fragments (3.2 kb and 1.9 kb) containing *BA* gene; lane 2, non-transformed tobacco plant DNA; lanes 3 ~ 7, *BtS29K*-BA transgenic plants.

The pattern of hybridization with *Bt* gene probe suggests that the insect-resistant gene has been inserted into the genome of these 5 plants as single copy. The different sizes of the hybridization bands for different plants (Fig.5(a)) reflect the different integration sites of *Bt* gene on the plant genomes.

The kanamycin resistance of the T_1 progeny of these 5 transgenic plants was analyzed and a typical

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3:1 segregation was observed (data not shown). This also confirms the conclusion of single copy insertion as revealed by Southern analysis.

2.5 Western blot analysis of transgenic plants

Western blot analysis of proteins from the transgenic plants was performed using Cry1Ac antiserum and API antiserum respectively. The results of the Western blot immuno-detection shown in Fig. 6 indicate that the proteins of the transgenic plants can specifically react with antisera of Cry1Ac and API respectively, suggesting that these two proteins are expressed in the analysed plants. The average expression level of the two proteins was estimated using Imagemaster 1.0 (Pharmacia), it is about 3.2 μg per gram fresh leaf, corresponding to 0.16% of the leaf total soluble proteins, for Cry1Ac and 4.9 μg per gram fresh leaf, corresponding to 0.25% of total soluble protein, for API.

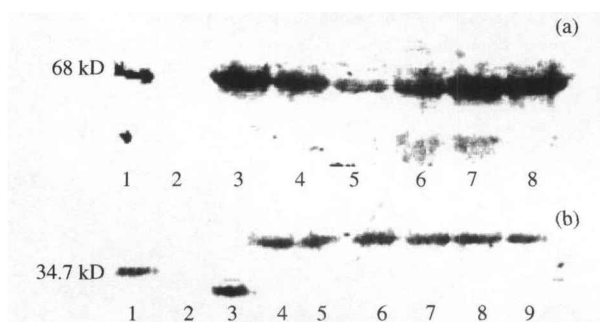

Fig. 6. Western blot analysis of pBS29K-BA transformed tobacco plants. (a) Detection of Cry1Ac. Lane 1, 0.1 μg Cry1Ac protein produced in *E. coli*; lane 2, non-transgenic plant; lane 3, *BtS29K* transgenic plant; lanes 4 ~ 8, *BtS29K-BA* transgenic plants. (b) Detection of API protein. Lane 1, a protein marker of 34.7 kD; lane 2, non-transgenic plant; lane 3, natural API protein (~20 kD, 0.1 μg); lane 4, BA transgenic plant; lanes 5 ~ 8, *BtS29K-BA* transgenic plants.

Since the molecular weight of the protein reacted with the antiserum against Cry1Ac does not show much difference with that of Cry1Ac produced by *E. coli*, at present, we cannot tell whether the signal peptide fused to Cry1Ac is processed or not. However the Cry1Ac produced by transgenic plants has definitely insecticidal activity. In comparison with the molecular weight of natural API protein, the molecular weight of the fusion protein API-BA produced in transgenic plants is twice as that of the natural one, suggesting that the fusion protein API-BA was not processed to form two natural API. Therefore the strategy used to construct the fusion *API-BA* gene needs to be improved. However, results shown in Figs. 3, 4 and 7 prove that this fusion protein still has proteinase inhibitory activity and insecticidal activity.

2.6 Proteinase inhibitory activity of *API-BA* transgenic plants

Proteinase inhibitory activity of pBS29K-BA transgenic plants was determined using the partially purified soluble proteins of the two transgenic plants and the results are presented in Fig. 7. Under the condition of measuring enzyme activity, about 50% of the trypsin activity was inhibited by proteins extracted from the two transgenic plants, while very weak inhibition could be observed at higher protein concentration if the proteins were isolated from non-transformed plants.

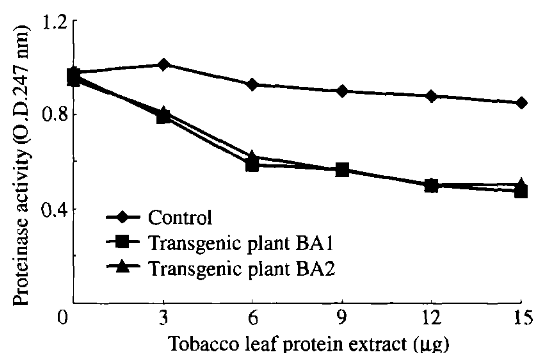

Fig. 7. Determination of proteinase inhibition activity.

Insect-resistant transgenic plants transformed with two insect-resistant genes, the *Bt* toxin gene and cowpea trypsin inhibitor (*cpti*) gene, have been reported recently[3,20~22]. However this is the first report of transgenic tobacco plants expressing a chimeric gene *BtS29K* which encodes the activated Cry1Ac protein and *API-BA*, a fusion protein gene of API-A and -B, whose gene structure and type are different from those reported. The results presented here demonstrate that co-expression of fully activated Bt toxin protein and a proteinase inhibitor in transgenic plants can assure that the latter one will not inhibit the function of the former and allow the two different types of insect-resistant proteins to fully function in the insect gut. However the processing and destination of these two proteins remain to be studied.

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