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Full Length Research Paper

Insect-resistance and high-yield transgenic tobacco obtained by molecular breeding technology

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The modified synthesized *VHb* gene and insectidal gene (GFM*cryIA*) were transferred to tobacco plants by *Agrobacterium*-mediated transformation. The bivalent genes were inserted successfully into the tobacco genome and detected by PCR amplification. Southern blot and Western blot analyses showed that *VHb* gene was expressed in the transgenic plants. Toxicity assay indicated that insectidal gene expressed pesticidal toxin protein. The net weight of transgenic tobacco plants exceeded that of non-transgenic ones by 8%. Compared to non-transgenic tobacco plants, transgenic plants appeared to be high-yielding, insect-resistant varieties.

Key words: *VHb* gene, insectidal gene, transgenic plant, pest resistance, yield.

INTRODUCTION

Expression of foreign genes in transgenic plant using molecular breeding technology, which was used to produce insect-resistance and high-yield transgenic plant, had great significance for agricultural sustained development. Significant advances have been made in insect-resistant gene *cry* engineering (Cui and Guo, 1998; Ni et al., 1998; Yan, 2003). Insect-resistant trangenic cotton, harbouring the modified, synthesized gene *cry*, has already been commercialized (Yan, 2003). The bacterium *Vitreoscilla* sp. is able to synthesize haemoglobin and adapt itself to a low-oxygen environment.

Because of its ability to bind oxygen, *Vitreoscilla haemoglobin* (VHb) is very important for the life cycle of the aerobic organism and has potential value in transgenic engineering (Wu et al., 2004a). In accordance with the amino acid sequence of the protein, the modified *vgb* (*vgbM*) harbouring the full-length sequence of 450 bp was synthesized according to plant bias codon (Wu et al., 2004b). In this paper, the plant expression vectors harbouring the bivalent *vgbM* gene and insectidal gene were successfully constructed. We assumed that, by transferring the bivalent gene to the host plants, we could obtain transgenic plants with high yield and resistance to insects.

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MATERIALS AND METHODS

Bacterium and gene materials

Escherichia coli DH5α and *Agrobacterium tumefaciens* LBA4404 were obtained from our laboratory collection. pG4AB harbouring the gene *Bt* expression cassette was described by Wu et al. (2001), pGSVHB harbouring the gene *vgbM* by Wu et al. (2004a) and pGBIF4ABC harbouring fused insectidal gene (*GFMcryIA* and *CPTI*) expression cassette by Wu et al. (2001); they are all preserved in our laboratory.

Plant and animal materials

Seeds of *Nicotiana tabacum* NC89 were kept in our laboratory. Leaves of NC89 were collected from plants grown under sterile conditions. Transformation of tobacco by *A. tumefaciens* LBA4404 was described by Li et al. (2006). *Heliothisarmigera* (3 days old) was obtained from the Plant Protection Institute (Chinese Academy of Agricultural Science). Tobacco plant genomic DNA was isolated according to Sambrook and Russell (2002).

Main reagents

Most reagents were purchased in China. Restriction enzymes and T4 DNA ligase were ordered from MBI (Beijing), DNA marker form Tiangen (Beijing) and protein marker from MBI. Primers were synthesized by Saibaisheng (Beijing).

The labeled probe kit, which was used for polymerase chain reaction (PCR) and Southern blots, was bought from Promega (Beijing). According to the full-length sequence of fused insectidal

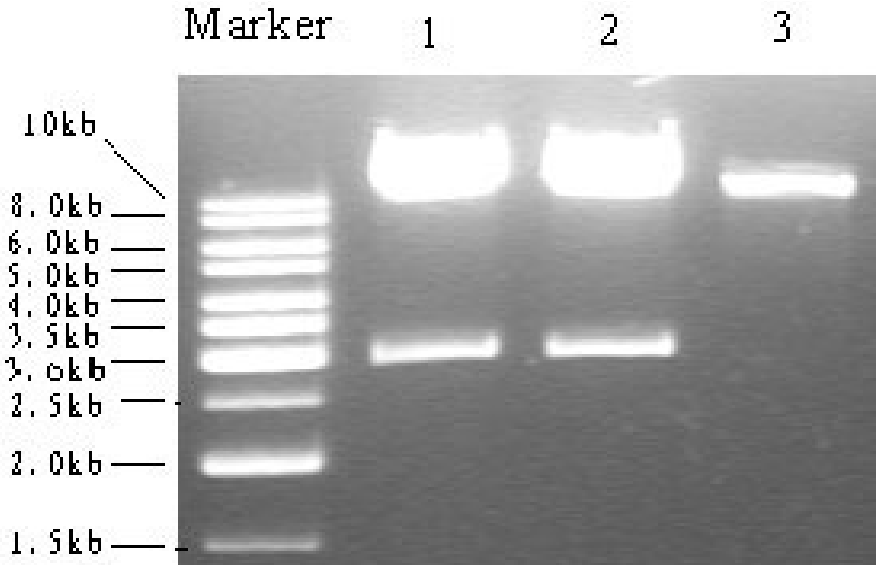

Figure 1. Restriction endonuclease analysis of plasmid pGBI4ASVHBBt. Lane 1: PGBI4ASVHBBt/HindIII; lane 2: PGBI4AB/HindIII; lane 3: PGBI4ASVHB/HindIII.

gene and *vgbM*, we designed four primers to identify whether they are integrated into the genomic DNA of the transgenic plants as follows:

p1: 5'-TACAATAACAATGGACTGC-3'
p2: 5'-AGATGTCCATCAAGTGGGT-3'
P3: 5'-CATGCCATGGGCCTTGATCAACAGACTATC-3
P4: 5'-CCCAAGCTTACTCAACAGCTTGAGC-3'

Construction of plant expression vector harbouring bivalent gene

pG4SVHB and pGBI121 were digested with *Hin*dIIIH and *Eco*RI, and *VHb gene* expression cassette (1.6 kb) and pGBI121vector fragment (10 kb) were collected, then pGBI4ASVHb (11.6 kb) was constructed by cloning *VHb gene* expression cassette into pGBI121vector, pGBI4AB and pGBI4ASVHB were digested by *Hin*dIII, *Bt gene* expression cassette (1.6 kb) and pGBI4ASVHb fragment (11.6 kb) were collected, plant expression vector pGBI4 ASVHBBt was constructed by inserting *Bt gene* expression cassette into pGBI4ASVHB.

The survey of transgenic tobacco plants

PCR reaction conditions were 94: for 3 min, then 94: for 30 s, 50: for 1 min and 72: for 1 min for 30 cycles, finally extension at 72: for 10 min and Southern blot was described by Li et al. (2006). Toxicity assay of transgenic tobacco plants was performed by using 3-day-old *Heliothisarmigera* and was inspired by Wu et al. (2004)

RESULT

Construction of plant expression vector harbouring bivalent gene

The expression cassette harbouring *vgbM* (1.6 kb) contained cis-acting elements 35S promoter with two enhancers, Ω leader sequence, Kozak sequence, and poly terminators and Nos terminator. The expression vector harbouring the insectidal *gene* (14.8 kb) contained the splicing and processing elements and Poly (A) sequence besides the cis-acting elements above. The plant expression vector (pGBI4ASVHBBt) bearing the bivalent gene contained the expression cassette harbouring *vgbM* and that holding the insectidal gene. pGBI4ASVHBBt, pGBI4AB and pGBI4ASVHB were digested with *Hind*Ш, the 3.2 kb fragments harbouring *vgbM* and 11.6 kb expression cassette harbouring the insectidal gene are identified in Figure 1. The orientation of *vgbM* was assayed by digesting pGBI4ASVHBBt with *Bam*HI. The result indicated that the plant with high-expression vector was successfully constructed. The plasmid pGBI4ASVHBBt was described in Figure 2.

Molecular assay of the transgenic tobacco plants

Thirty-six transgenic tobacco plants resistant to kanamycin were obtained by *Agrobacterium*-mediated transformation of tobacco plant discs. Following extraction of the genomic DNA from the transgenic tobacco plants, PCR detection was conducted using primers 1, 2, 3 and 4 (Figure 3 and 4). The results of PCR Southern blotting are also shown in Figures 5 and 6. These results showed that 32 transgenic plants were positive for *vgbM* and insectidal gene.

Western blot assay of the expression of *vgbM* in transgenic tobacco plants

The result of the Western blot assay between the trans-

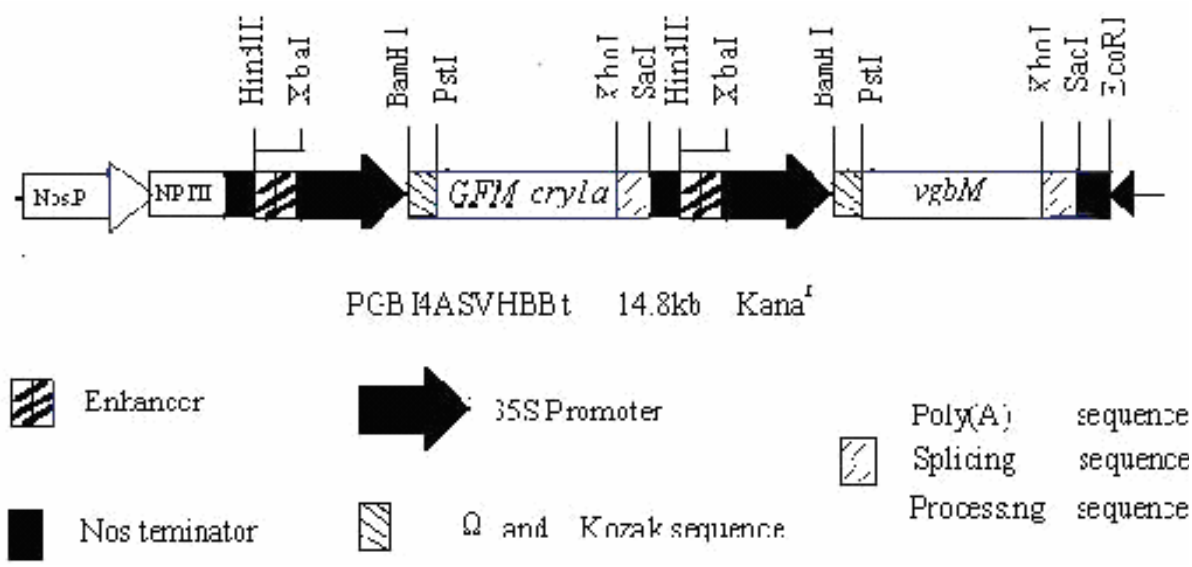

Figure 2. The structure of expression vector pGBI4ASVHBBt.

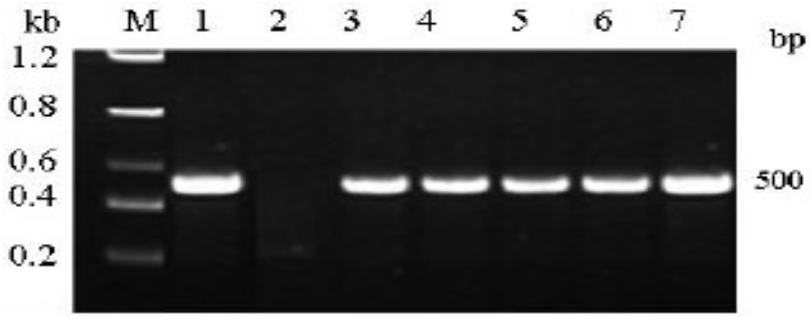

Figure 3. PCR amplification detection of *vgbM* gene in transgenic tobacco. Lane 1: Positive group; Lane 2: non transgenic group; Lanes 3-7: the transgenic tobacco plants.

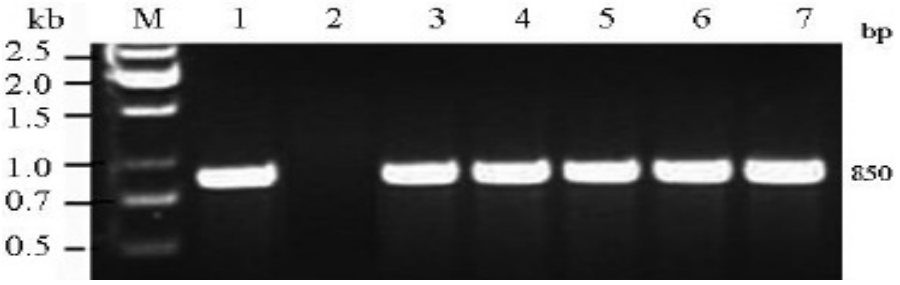

Figure 4. PCR amplification detection of insectidal *gene* in transgenic tobacco. Lane 1: Positive control; Lane 2: non transgenic group; Lanes 3-7: the transgenic tobacco plants.

genic tobacco plants and the wild type showed that the *vgbM* was expressed in the transgenic tobacco plants and parts of the western blot assay were described in Figure 7.

Detection of net weight of the transgenic tobacco plants

To test the expression of *vgbM* in transgenic tobacco

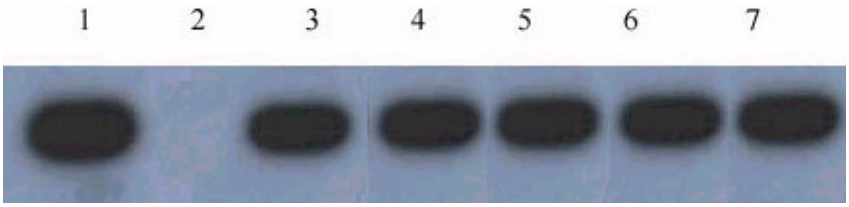

Figure 5. Southern blot analysis of *vgbM gene* in transgenic tobacco. Lane 1: Positive group; lane 2: non transgenic group; lanes 3-7: the transgenic tobacco plants.

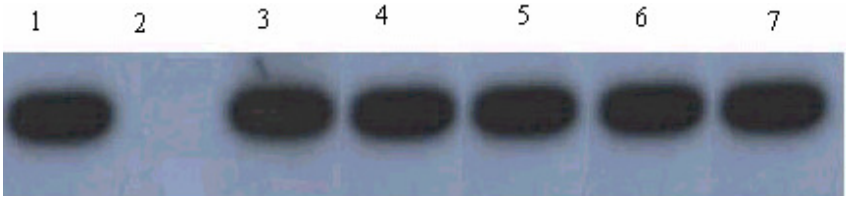

Figure 6. Southern blot analysis of fusedly-insectidal gene in transgenic tobacco. Lane 1: Positive group; lane 2: non transgenic group; lanes 3-7: the transgenic tobacco plants.

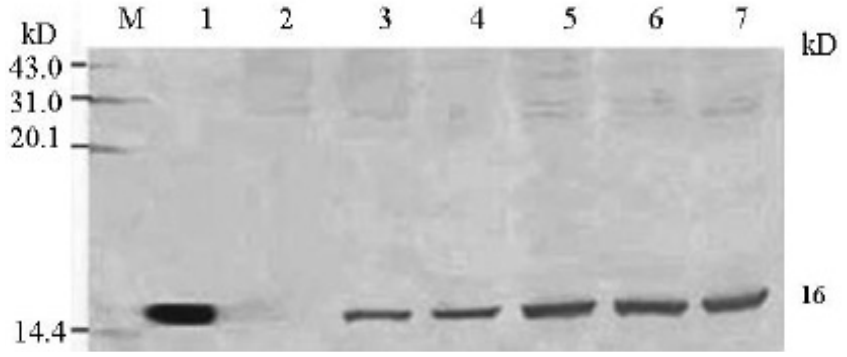

Figure 7. Western blot analysis of VHb *gene* of transgenic tobacco plants. Lane 1: Positive group; lane 2: non transgenic group; lanes 3-7: transgenic tobacco plants. The results of western blot corresponded to the that of PCR and PCR southern blot in the Figures from 3 - 7.

plants, average weights of 32 transgenic plants and wild-type tobacco plants were analyzed. The result showed that the net weight of transgenic tobacco plants was 108% higher than that of the wild type (Table 1), indicating that not only *vgbM* was expressed in host tobacco plants, but their yield also increased.

Toxic assay of transgenic tobacco plant towards Heliothisarmigera

The toxicity assay indicated that most of the transgenic plants showed high resistance to *Heliothis armigera*. Among 32 transgenic tobacco plants, 46.8% of the transgenic plants showed high resistance to the insect, 34.5% exhibited medium resistance and 18.7% low resistance (Table 2).

Analysis of segregation of insectidal genes

T_0 tobacco plant seeds were grown on medium containing kanmycin (200 μg/ml), χ^2 analysis indicated that 27 transgenic plant exhibited Mendal's law of inheritance, 5 did not show Mendal's law (data not shown).

DISCUSSION

Codon bias, characterized by differences in tRNA abun-

Table 1. Dry weight of tobacco plants.

The type of plants	The number of plants	The average dry weight of each plant	The proportion of dry weight of transgenic plants to that of wild ones
transgenic	32	3.543	107.9%
nontransgenic	30	2.744	100%

Table 2. Evaluation of insect resistance to insect of leaves from transgenic tobacco plants.

Resistance type	Corrected ratio of death larvae	Numbers of high resistance to insect	The propotion of resistant plants
High-resistant	> 80%	15 *	46.8%
Medium-resistant	50 - 80%	11 **	34.5%
Low-resistant	< 50%	6 *	18.7%
Resistant		32	100%

Duncan's test (LSR); *, significance level ($p < 0.05$); **, extreme significance level ($p < 0.001$).
Plants with high resistance were defined as those with a corrected proportion of dead larvae > 80%.
Plants with medium resistance were defined as those with a corrected proportion of dead larvae = 50 - 80%.
Plants with low or no resistance were defined as those with a corrected proportion of dead larvae < 50%.
Corrected proportion of dead larvae = [(the death ratio of larvae as transgenic plants) - (the death ratio of control group larvae)/1 - (the death ratio of control group larvae)]$\times$ 100.

dance of different codons *in vivo*, is common (Dikshit et al., 1990; Jelenkovic et al., 1998; Wang et al., 1998). Therefore, the modified gene *vgb* was synthesized (named as *vgbM*) to enhance the expression of *vgbM* in host plants and eventually increase the growth rate and yield of transgenic plants (Wu et al., 2004b), due to the enhancement of oxygen and ATP supply by VHb. Oxygen and ATP are both involved in the process of chlorophyll biosynthesis (Dikshit et al., 1990). Thus, thanks to this chlorophyll increase and to the subsequent enhancement of leaf photosynthesis, transgenic tobacco plants grew quickly and their yield increased. Significant advances in insect resistance engineering of transgenic plants have been achieved in the past two decades, and the good prospects for application of *Bt* transgenic insect-resistant plants (Wang and Guo, 1999; Li et al., 2006). The gene can be transferred to host plant by molecular breeding, therefore, to create new varieties, the expression of foreign gene was directly regulated by the interaction between cis-acting element (promoter, enhancer, etc) and the trans-acting element. The expression cassette of the *VHb* and *GFMcryIA* gene contained such cis-acting elements as followed: 5' CaMV35S promoter and enhancers double element, 5' Ωsequence and Kozak sequence, 3'poly (A), processing and splicing sequence, 3' poly termination sequence and Nos terminator (Dikshit et al., 1990; Wu et al., 2001). In host plant, the foreign genes were expressed more efficiently and functioned much better (Cui and Guo, 1998 a, b; Guo et al., 1999; Zhao et al., 2005). Results show that transgenic tobacco plants were obtained by *Agrobacterium* mediated transformation; 32 of them were identified as insect-resistant and high-yielding transgenic plants, which laid a good foundation in promoting the production of new plant varieties with high and stable yields as well as insect resistance.

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