转 *Bt-CpTI-GNA* 基因棉花的研究

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摘要：采用农杆菌介导法将外源三价抗虫基因(*Bt-CpTI-GNA*)导入常规棉花品种中，获得转基因再生株，分子检测表明外源基因已在棉花体内表达，并遗传给后代材料。PCR 分子检测与转化的标记基因和外源目的基因抗性三者极有规律性。其所携带的基因在转基因棉花中有分离现象，这可能是外源基因整合到受体棉株体内“基因沉默”而引起所致。

关键词：棉花；农杆菌介导转化；*Bt-CpTI-GNA*；抗虫性；标记基因

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Study of Transgenic Cotton Carrying *Bt-CpTI-GNA* Genes

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Abstract: Foreign genes (*Bt-CpTI-GNA*) resistant to cotton insects were introduced into conventional cotton cultivars by *Agrobacterium tumefaciens*-mediated transformation. Regenerated plants have been obtained. The results of molecular detection indicated that the foreign genes had been transferred into cotton plants. The results of PCR molecular detection, marker genes and foreign target-gene with insect-resistance were quite regular. Some of their progenies' segregation coincided with Mendel's regularity of segregation, but some deviated. This phenomenon may be due to "gene silence".

Key words: cotton; *Agorbacterium tumefaciens*-mediated transformation; *Bt-CpTI-GNA*; insect-resistance; marker gene

1 Materials and Methods

1.1 Construction of Genes

Insect-resistant genes (*Bt-CpTI-GNA*) were constructed by 601 team, Genetics and Development Institute, Chinese Academy of Sciences. Bt and *CpTI* were fusion protein, *NPT*Ⅱ marker gene. The map of gene structure is shown in Fig. 1. The actual inserting sequence is about 11 kb, which contained *BT-CpTI* fusion gene and *GNA* gene and *NPT*Ⅱ gene between the left and right boundary of plasmid pCRPSBCK-OMGNA.

The whole length of the nucleotide sequence of target gene and deduced amino acid sequence is of 2205 bp of modified *Bt-CpTI* gene sequence, which coded 735 residues of amino acid. *GNA* genes with whole length of 484 bp were obtained by RT-PCR, which could code 157 amino acids and two termination condos.

There existed 23 amino acid signal peptides

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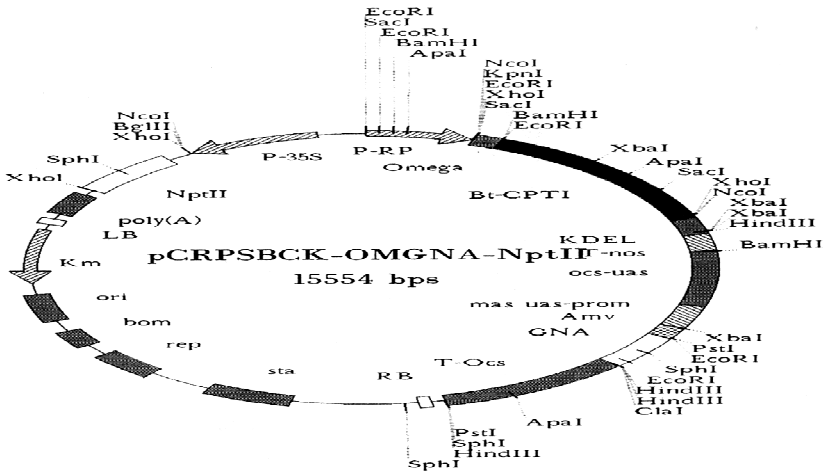
at N'-termination, 29 amino acid's C' sequences at the end, GNA sequence coded 105 amino acids in the middle.



pCRPSBCK-OMGNA-NptII was constructed by inserting the KpnI(T_4Pol) and SalI(Klenow) fragment of p7RPBTCM into the SmaI site of p7RPG2300

Fig. 1 Plasmid-pCRPSBCK-OMGNA-NptII map

1.2 Transformed cotton recipients

Jihe-713, Xinluzhong 9, Jihe-312, and color cotton cultivars etc.

1.3 Transformation of cotton foreign genes

Transformation of triple insect-resistant cotton foreign genes was accomplished by Cotton Research Institute, Shanxi Academy of Agricultural Sciences. In this study, cotton was transformed by *Agobacterium tumeficiens*-mediated, the manufacture procedures were as follows[1-2].

1.3.1 *Preparation of cotton recipients.* Seeds removed lint in sulphuric acid solution were washed with running water, then soaked in 10% H_2O_2 for 3～4 h, washed with steriled water for 2～3 times, and soaked in sterile water at 25～28 ℃ for 24 h for culturing, removed the seed coats, and placed on 1/2 MS medium. After germination, 3～5-day-old seedlings grown in darkness at 25～28 ℃ on 1/2 MS media were used to separate with hypocotyl segments in 0.5～1 cm length in the steriled condition.

1.3.2 *Preparation of Agrobaterium tumefaciens.* 20 ml liquid media YEB pasteurized in high pressure cooker was added to appropriate antibiotics(Kan 25 mg · L^{-1}, rifamycin 50 mg · L^{-1}) infected by single colonia placed on the plate, then they were placed on the 28 ℃ permanent shaking table (150～299 r · min^{-1}) overnight.

1.3.3 *Procedures of transformation.* The hypocotyls segments were soaked in *Agrobaterium tumefaciens* solution, which was diluted to a density of 5×10^7 bacteria · ml^{-1} prior to use, for 1～8 min, co-cultured on co-cultivation medium(MS salts, B5 vitamin, 100 mg · L^{-1} myoinositol, 30 g · L^{-1} glucose, 0.1 mg · L^{-1} 2,4-D and 0.1 mg · L^{-1} KT, pH 5.8) covered with filter for 48 h.

1.3.4 *Obtaining of transgenic regenerated plants.* After two-day's co-cultivation, the hypocotyl segments were cultured on selective medium supplemented with 50～100 mg · L^{-1} Kan, 500 mg · L^{-1} cefotaxime and other components same as co-cultivation medium for 40～50 d to induce transformation calli, then the calli were cultured on the same medium for 25～30 d for selection. After than the calli were the transferred onto inducement medium (other components were same as co-cultivation medium), without Kan, cefotaxime, 2,4-D and KT for subculture 2～5 times to induce embryonic calli. 5～6 months later, the well-developed embryoids were transferred onto differential medium (MS salt, B5 vitamin, double KNO_3, added to 1.0 g · L^{-1} glutamin, 0.5 g · L^{-1} aspragine, pH

5.8, without NH_4NO_3) to produce somatic embryos, subculture once a month. At the stage of 3～4 leaves, the seedlings were grafted into healthy stocks on the pots, kept moisture for 7 d, the grafted plants could grow normally.

1.3.5 *Detection of transformed progenies.* A) Detection of target gene. First, it was conducted with bollworms indoors. During the growth period of transgenic cotton, the second instar larvae of bollworms were fed with fully expanded young leaves on the top for 4～6 d. According to the development of larvae and their situation of eating leaves, detection was conducted. Second, they progenies were detected by molecular analysis. Inserting sequence in the site of plant cells whether integrated into chromosome, chloroplasts, mitochondria or existed in non-integrated form and defined method were detected by molecular analysis. Extracted DNA were amplified by PCR, and the target genes, *Bt* (amplified band 771 bp), *GNA* (amplified band 415 bp by electrophoresis). Extracted DNA of leaves in transgenic plants was carried out according to CTAB method (Zolan M E. et al). DNA digestion of plants, electrophoresis and transferring to HNO_3 cellulose membrane were conducted according to conventional methods. After pCRPSBCK-OMGNA-NptⅡ was digested with *BamHI or Hind* Ⅲ, and electrophoresis was conducted, either *Bt-CpTI* gene or *GNA* gene fragments were got as a probe, marked with [α-32p] dCTP, taking random primer (Progega reagent box). Southern hybridization was conducted according to the method of molecular clone[13].

B) Detection of marker gene. During cotton growth period, cotton leaves were painted with Kan at the concentration of 5.0-7.5 g · kg^{-1} (tested in the rooms). First, the 3rd leaves of F_0 on the top were painted with 5.0-7.5 g · kg^{-1} Kan. Five days later, leaves were identified according to the color, green leaves were transgenic plants, yellow leaves were non-transgenic plants. Second, F_1, F_2 etc were painted with Kan. during growth period. After being harvested, 50-100 seeds sampled at random were sowed in growth medium contained 0.75-1.00 g · kg^{-1} Kan. Four days later, leaves were identified according to the sterile leaves color, green leaves were transgenic plants, yellow leaves were non-transgenic plants.

2 Results and Discussion

2.1 Names of target genes, carriers, source, structure, character and security

The construction of plasmid pCRPSBCK-OMGNA-NptⅡ were modified *Bt-CpTI* fusion protein gene obtained by PCR of three primers method added to DKTI signal peptide sequence and endoplasmic reticulum (ER), detection signal sequence KDEL and cloned in pSBCKm plasmid. pSBCKm were digested with *BamH2* and flatted with Klenow and then digested with *Cla I* again. Collected target fragments were inserted into p7RPnos expressing carrier to obtain p7RPBtCKm. Expressing carrier p7RP2300 of all-purpose plants was further constructed, which contained important expression parts of pCAMBIA2300 carrier, i. e. left and right boundary sequence, selective marker Kan gene for plants and bacteria. p7RPBtcKm was digested with double enzymes *Kpn I/Sal I* to obtain fragments contained modified *Bt-CpTI* fusion protein which were inserted into the site of *KpnI/ Sal I* of p7RP2300 to obtain p7RPBCKan. pOGO contained *GNA* gene expression structure regulated by compound promoter and AMV translation enhance sequence. pOGO was digested with *BamHI*, flatted with *Klenow*, and digested with *Xhol* again to obtain target gene fragments, which were inserted into p7RP2300 to express carrier, and to obtain p7RPG2300. In order to obtain expressing carriers of triple insect-resistant genes, p7RPBtCKm was further digested with *KpnI* and its sticky ends were flatted with T4 polylase, then digested with *Sal I*, and flatted the ends. Gene fragments collected were inserted into the site of SamI of p7RP2300 to obtain pCRBSBCK-OMGNA, which con-

tained triple insect-resistant genes (*Bt*, *CpTI* and *GNA*) regulated, respectively, by PRP and double promoters (OCS uas)$_6$(mas), which contained Ω and AMV translation enhancer sequence, respectively.

pCAMBIA2300 series derived from pPZP[4] was a part of mimi-Ti plasmid for dualcarrier-system, whose replication starting sites had two, replicated ColE1 in *E. coli*. (derivedfrom *E. coli* plasmid pBR322), respectively, replicated replication starting site of pVS1 in *Agrobacterium*[4]. When *Agrobacteria* were used to transform plants, only T-DNA region could be integrated into plant genome, the replication starting site of carrier located beyond T-DNA region, therefore, it was impossible to be transferred into plant cells[5].

The bacterium selective marker of PCABIA2300 was Npt Ⅱ, which coded Kanr, it hasn't been reported that products coded by it are toxic -by-effect to animals. Bejing located outside T-DNA region[6], it couldn't be transferred into plant cells. Plant selective marker of PCAMBIA 2300 was also used as Kan selective marker gene.

T-DNA of PCAMBIA 2300 carrier series derived from T-DNA region of Ti-plasmid pTi37, creating tumor genes and sequences which were irrelevant to T-DNA transference had been removed, only maintained right boundary sequence (RB) and left boundary sequence (LB) which were necessary for T-DNA transference, and inserted Npt Ⅱ gene as plant selective marker. In the course of construction, *LacZ* gene existed in pCAMBIA 2300 was destroyed. RB and LB were distinguishing sequence of exonuclease at T-DNA transference, they didn't code any gene products[5].

2.2 Information of each fragment of inserted regions in carriers

2.2.1 *Size and function of promoter and terminator and names of donor organisms.* PRP promoter controlling expression of *Bt-CpTI* fusion gene in plasmid derived from cotton curly leaf virus genome and lay in upstream promoter region of replicating protein gene and coat protein gene, 436 bp long, which contained virus replication origin, could promote gene expression in parasites such as cotton , tobacco tomato, okra, French pea and in the body of white fly. Reports on the damage of this promoter itself haven't been seen yet. The terminator used was nos3′ sequence, about 260 bp long, which derived from nopaline synthetase gene's 3′-termination sequence of Ti-plasmid's T-DNA region. All the gene expression regulation members mentioned above have been proven to have no pathogenicity, and widely applied to plant genetic engineering.

The promoter controlling *GNA* gene was a chimeric OM promoter derived from *Agrobacterium's* Ti-plasmid, which is composed of three octopine synthetase gene (OCS) upstream activating sequeances and Mannopine synthetase gene (MAS) promoter[(OCS)3 MAS], 320 bp long. The terminator used was nos3′ sequence, about 1200 bp, which derived from OCS's 3′-termination sequence of Ti-plasmid′s T-DNA region. All the gene expression regulation members described above have been widely applied to plant genetic engineering. Its damage hasn't been reported yet.

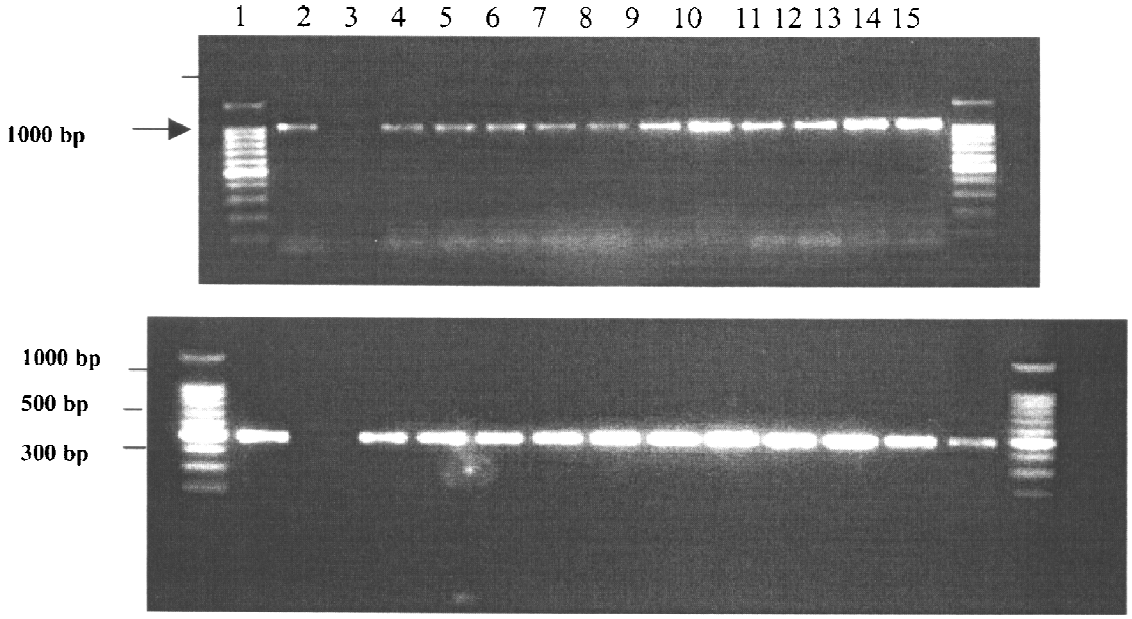
2.2.2 *The size and function of marker gene, report gene and donor organisms.* Neomycin phosphotransferase (Npt Ⅱ) as plant selection marker, which also called APH(3′) H (Aminoglycoside antibiotics 3′-phosphotransferase Ⅱ) derived from Npt Ⅱ gene code sequence of transponson Tn5 in *E. coli*, 800 bp long. While APT existing, Npt Ⅱ could make aminoglycoside antibiotics phosphorylation and out of activity. Gene expression regulation members of Npt Ⅱ gene in pCRPSCK-OMGNA-Npt Ⅱ derived from NOS of Ti-plasmid′s T-DNA region. All the genes and members mentioned above have been proven to have no pathogenicity.

2.2.3 *Other expression adjusting-controlling sequences and their origin.* Ω factor and SKTI

signal peptine code sequence were added to 5′-termination of *Bt-CpTI*; protein endoplasmic reticulum (ER) detention signal sequence KDEL were added to 3′-termination, among which Ω factor derived from a fragment of gene expressing enhance sequence of AMV, SKTI and KDEL were obtained from plant genome by amplification, according to the known sequence.

2.3 Molecular detection of foreign gene in transgenic cotton plants

Bt-CpTI and GNA have been proven to be integrated into cotton chromosome by PCR analysis for transgenic cotton (Fig. 2). It was confirmed that there was only one copy for each of *Bt-CpTI* and *GNA* based on Southern hybridization analysis.



upper PCR product of Bt gene in transgenic cotton plants contained triple genes

lower PCR product of *GNA* gene in transgenic cotton plants contained triple genes

Notes: 1, 15 : 100bp DNA Marker; 2: plasmid amplification-positive control; 3: non-transgenic cotton-negative control; 4 - 14: amplified band of transgenic cotton plants

Fig. 2　Electrophoresis diagram of transgenic cotton plants with insect-resistant genes (*Bt-CpTI-GNA*) amplified by PCR

2.4 Genetic characteristics of regenerated plants

The result shown in Table 1 was that Bt amplified bands and *GNA* amplified bands in transgenic regenerated cotton plants differentiated from the same callus camp performed differently, which was detected by PCR. 11 plants regenerated from five callus camps, respectively, only the result of No. 3D-8-713 and No. 22-713 was same, while that of other three callus camps was different. Three plants regenerated from 713-BCG-58, two plants of which contained both Bt and *GNA* amplification bands of PCR, one plant of which contained nothing. Three plants were regenerated from 5D-15-713, only one plant of which contained both Bt and *GNA*

Table 1　Genetic characteristic of Fo regenerated plants with insects-resistantgenes (*Bt* + *CpTI* + *GNA*) derived from the same callus camp

materials	Acc. No. of plants	Bt	*GNA*
5D-15-713	1991	V	V
	1002	V	-
	1027	-	V
713-BCG-58	1996	V	V
	1039	V	V
	1030	-	-
13-713	2013	V	V
	1009	-	V
3D-8-713	1012	V	V
	1997	V	V
22-713	986	V	V

Note: V: indicated there existed amplified bands detected. -: indicated there existed no amplified bands detected.

amplified bands, as to the other two plants, one plant contained only Bt amplified bands but no *GNA* amplified band. The other plant contained *GNA* amplified bands without Bt amplified bands. Two plants were regenerated from 13-713, one plants of which contained both Bt and *GNA* amplified bands, one plant of which contained only *GNA* amplified band without Bt amplified band. All these characters confirmed that the foreign genes to be introduced into recipients resulted in the difference of gene expression in transgenic cotton plants. On one hand, foreign genes were inserted into chromosome at random; on the other hand, it might be caused by "gene silence"; In addition, the transgenic plants derived from the same callus clamp, not from the same cell.

Table 2 Insect-resistance of F_0 regenerated plants derived from the same callus camp

materials	Acc. No. of plants	insect detection July 25	insect detection July 12	Kan green	Kan yellow
14-713	900	+	++	V	
	1010	+	+	V	
1-4D-321	961	++	+	V	
	1019	+	+		V
2-713	978	+	+	V	
	1031	+	+		V
22-713	989	++	+	V	
	992	+	+	V	
	1044	++	+	V	
	1060	++	+	V	
3D-8-713	931	+++	+++	V	
	1012	++	++	V	
	1041	+++	++	V	
	1046	++	+++	V	
	1048	+++	+	V	
	1054	+	+	V	
9-713	1017	+	+	V	
	1021	+	+	V	
	1028	+	+	V	
713-BCG-58	1036	+++	+	V	
	1058	+	+	V	

Notes: V: indicated that transgenic cotton leaves painted with Kan, showed green or yellow. + : indicated that the high insect-resistance type of transgenic cotton. ++ : indicated moderate insect-resistance type. +++ : non-insect-resistance type.

Observing the insect-resistance, only one clamp 9-713 of three clone clamps shown in Table 2 made no difference for insect-resistance, which was consistent with that of marker gene, classified as high insect-resistance. There existed slight differences between 14-713 and 22-713, which were high insect-resistance and moderate insect-resistance, respectively, their marker genes both were green. There were a little bit difference between 1-4D-321 and 2-713, but the performance of marker genes was obviously different. Yellow color indicated that marker gene didn't express, but with high insect-resistance. The marker genes for 3D-8-713 expressed, but big difference for insect-resistance existed between them, which ranged from high insect-resistance to non-insect-resistance. On one hand, it might be due to irregular distribution of chromosome fragment's site caused by target gene in transgenic cotton plants; on the other hand, it

might be that though the target gene have been introduced into recipient plants, expressed toxicity hasn't reached the lethal level to cotton bollworm.

3 Summary

3.1 Some genes' no expression in transgenic cotton plants caused by different gene expression level, inserting site, and interaction among genes carried by transgenic cotton plants resulted in vague segregation in transgenic cotton plant of early generation. In late generation transgenic materials, the segregation was stable. In early generation transgenic cotton materials, besides marker gene, target genes were mainly used to detect, and low insect-resistant materials must be eliminated, or amount of selection and breeding work would be increased.

3.2 Transgenic cotton selection and breeding are different from conventional one, because it contains exogenous gene. Not only does it request foreign genes to express normally, and play the role of target gene, but also it requests transgenic cotton with conventional cotton yield, fiber quality and other characters. Therefore, in selection and breeding of transgenic insect-resistant cotton, the insect-resistance should reach over 90%, yield character is a key objective. Both of them should be taken into account, supplement each other, no one is dispensable.

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