Ubi1 intron-mediated enhancement of the expression of Bt cry1Ah gene in transgenic maize (Zea mays L.)

Article *in* Chinese Science Bulletin · October 2008

DOI: 10.1007/s11434-008-0379-1

CITATIONS
18

READS
75

6 authors, including:

Yuebing Wang
Nankai University
29 PUBLICATIONS **324** CITATIONS
SEE PROFILE

Zhihong Lang
Chinese Academy of Agricultural Sciences
29 PUBLICATIONS **214** CITATIONS
SEE PROFILE

Jie Zhang
1,137 PUBLICATIONS **13,149** CITATIONS
SEE PROFILE

Kl He
Chinese Academy of Agricultural Sciences
143 PUBLICATIONS **1,189** CITATIONS
SEE PROFILE

Chinese Science Bulletin

Ubi1 intron-mediated enhancement of the expression of Bt *cry1Ah* gene in transgenic maize (*Zea mays* L.)

WANG YueBing[1], LANG Zhihong[1], ZHANG Jie[2], HE KangLai[2], SONG FuPing[2] & HUANG DaFang[1]

[1] Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China;
[2] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

The *cry1Ah* gene was one of novel insecticidal genes cloned from *Bacillus thuringiensis* isolate BT8. Two plant expression vectors containing *cry1Ah* gene were constructed. The first intron of maize *ubiqutin1* gene was inserted between the maize Ubiquitin promoter and *cry1Ah* gene in one of the plant expressing vectors (pUUOAH). The two vectors were introduced into maize immature embryonic calli by microprojectile bombardment, and the reproductively plants were acquired. PCR and Southern blot analysis showed that foreign genes had been integrated into maize genome and inherited to the next generation stably. The ELISA assay to T_1 and T_2 generation plants showed that the expression of Cry1Ah protein in the construct containing the *ubi1* intron (pUUOAH) was 20% higher than that of the intronless construct (pUOAH). Bioassay results showed that the transgenic maize harboring *cry1Ah* gene had high resistance to the Asian corn borers and the insecticidal activity of the transgenic maize containing the *ubi1* intron was higher than that of the intronless construct. These results indicated that the maize u*bi1* intron can enhance the expression of the Bt *cry1Ah* gene in transgenic maize efficiently

Bacillus thuringiensis, *cry1Ah* gene, *ubi1* intron, intron-mediated enhancement, insect-resistant transgenic maize

Maize (*Zea mays* L.) is one of the most important food, feed and industrial materials. Spurred by the soaring oil price, many USA companies produce alcohol fuel from biotech maize. As a result, the prices of cereal crops especially maize are sharply rising, bringing huge shocks to China's maize cultivation and production. Genetic engineering technologies must be engaged to increase the yield of maize if the impending global maize shortage is to be avoided, and it is also the key way to resolve the civil cereal problems.

Since transgenic maize expressing insecticidal proteins was first commercialized in USA in 1996, the global area of insect-resistant transgenic maize (IR maize) has continued to soar, reaching 20.1×10^6 ha in 2006[1]. In 2007, maize has become the fast-growing biotech crop in USA, the yield increasing by a substantial 40%[2]. Now almost 10 countries have been the adopters of IR maize, benefiting a lot in their economy, ecology and social profits[3]. In the research of IR maize, the introduction of crystal genes from *Bacillus thuringiensis* (Bt) is the most widespread. Transgenic varieties of Mon810 (Monsanto) and Bt11, 176 (Syngenta) harboring *cry 1Ab* gene have been on the market for nearly 10 years[4]. The exploitation of IR maize in China is still in process, but most researchers applied *cry1Ac* or *cry1Ab/cry1Ac* hybrid genes as materials, as with the case of the commercialized Chinese Bt cotton. In terms of hybrid cultivation system in China, maize and cotton harboring the same insecticidal genes will bring huge resistant risk to the farmers.

The *cry1Ah* gene was a novel insecticidal gene cloned

Received March 3, 2008; accepted April 29, 2008
doi: 10.1007/s11434-008-0379-1
†Corresponding author (email: dfhuang@mail.caas.net.cn)
Supported by the National Natural Science Foundation of China (Grant No. 30500039), National Basic Research Program of China (Grant No. 2003CB114201) and Basic Scientific Research Foundation for Chinese Central Academy (Biotechnology Research Institute, CAAS)

from the *Bacillus thuringiensis* isolate BT8 [5], which exhibited higher toxicity to lepidopteron insects than any other *cry* genes. The toxic fragment of Cry1Ah was 82% similar to that of Cry1Ac. The *cry1Ah* gene has been patented (Grant No. 2004 1 0009918.9). The modified *cry1Ah* gene, optimized for expression in plants, was 86% similar to the primary one. The GC % of modified *cry1Ah* gene was increased from 37% to 48%. If we make use of new highly toxic genes owning state intellectual property in transgenic IR maize, we can overcome the issue of monotonous Bt genes efficiently, delay resistance risk evolved by the insect species, and lay basis for the commercialization of IR maize in China.

Most eukaryotic genes are interrupted by one or more noncoding intervening sequences (introns). In 1987, Callis et al.[6] first found that intron could enhance the expression of foreign genes, which was termed as intron-mediated enhancement (IME). Subsequently many studies showed that pre-mRNA intron-containing versions can exhibit dramatically high expression profiles[7], because intron splicing can influence almost all steps of mRNA metabolism including transcription, capping, RNA editing, pre-mRNA processing, translation and decay of mRNA products[8,9]. A series of plant vectors with 5 different intron insertions were constructed and introduced into maize immature embryonic calli by microprojectile bombardment. Histochemical and quantitative assays showed that GUS activity in maize calli bombarded with a construct containing the *ubi1* intron was higher than any other construct (Wang, unpublished). According to this result, the *ubi1* intron was inserted between the maize Ubiquitin promoter and *cry1Ah* gene in the construction of the plant expression vectors. The intronless and intron-containing constructs harboring the *cry1Ah* gene with the *hpt* gene as the marker were introduced into maize immature embryonic calli by microprojectile bombardment. Molecular assay to T_0, T_1 and T_2 generation showed that foreign genes had been integrated into maize genome and inherited to the next generation stably. And we also confirmed that the *ubi1* intron could enhance the expression of *cry1Ah* in transgenic maize efficiently.

1 Materials and methods

1.1 Plant Materials

Embryogenic callus cultures from Maize (*Zea mays* L.) hybrid line Q31×Z3 were prepared for microprojectile bombardment transformation. About 10 d after self-pollination, the immature maize embryos of Q31×Z3 were isolated and cultured on modified N6 medium (2,4-D, 2 mg/L; L-pro, 690 mg/L) to induce callus. Embryonic calli were selected for subculture at an interval of 15 d.

1.2 Enzymes and Reagents

Restriction endonucleases and the pMD18-T vector were purchased from TaKaRa. Hifi-KOD DNA polymerase was purchased from Toyobo. Plasmid purification kit was purchased from Qiagen. Jin-Biao Cry1Ab/Ac immune detection strips were purchased from Chongqing Jin-Biao Biotechnology Ltd. EnviroLogix QuantiPlate kit for Cry1Ab/Ac was used for ELISA assay. *Escherichia coli* JM110 was preserved in our laboratory.

1.3 PCR amplification for the maize *ubi1* intron

Genome DNA isolated from maize leaves was used as template in the PCR reaction. The PCR reaction was performed according to the KOD handbook: initial denaturation at 94℃ for 5 min; 94℃ for 1 min, 60℃ for 1 min, and 72℃ for 1 min, 35 cycles. The PCR primers are F1 (5′-GTACGCCGCTCGTCCTCCC-3′) and R1 (5′-CGGGATCCCTGCAGAAGTAACACCAAACAAC-3′). The restriction site of *Bam*H I was introduced to facilitate the next plant expression vector construction.

The PCR products were recovered and ligated to the pMD18-T vector at 16℃ overnight, and the white colony was selected for sequence confirmation, which was named pT-U.

1.4 Construction of plant expression vectors

Plasmid pT-U was digested with *Bam*H I and recovered the *ubi1* 1010 bp fragment. The *ubi1* was then fused upstream of the *cry1Ah* gene in sense orientation to acquire the expression vector pUUOAH. The plant expression vector pUOAH consisted of maize constitutive promoter Ubiquitin, modified *cry1Ah* gene, nos terminator and hygromycin phosphate transferase gene for hygromycin selection. Except the *ubi1* intron, all elements in pUUOAH are identical to pUOAH (Figure 1).

1.5 Plant transformation

The protocol used for transformation with microprojectile bombardment and regeneration of transgenic plants was previously described by Zhang et al.[10].

1.6 PCR and Southern blot analysis of transformed maize plants

Genome DNA was isolated from regenerated maize

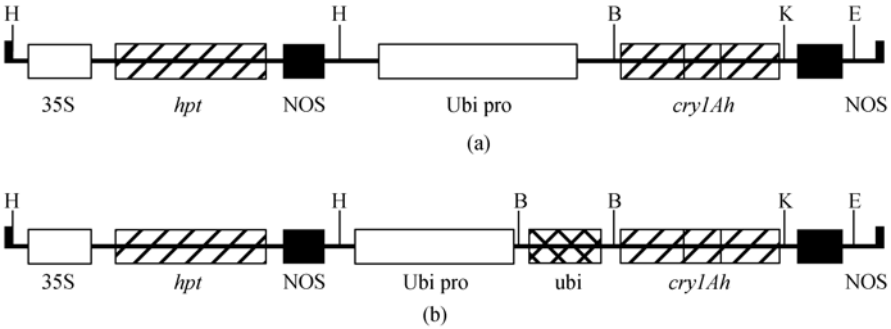

Figure 1 Construction of plant expression vectors. (a) pUOAH; (b) pUUOAH. H, *Hin*d III; B, *Bam*H I; K, *Kpn* I; E, *Eco*R I.

leaves by CTAB method[11]. Primers specific to *cry1Ah* gene were used for PCR. The primers are F2 (5′-TTCACCGCAACTGAGACTTACATC-3′) and R2 (5′-GGTTCTATACACGCCCTGACCTAG-3′). The amplified products were 684 bp. The PCR reaction was 35 cycles at 94℃ for 1 min, 56℃ for 1 min, 72℃ for 1 min.

Maize genomic DNA was digested with *Hin*d III, separated on 0.8% agarose gel and transferred to nylon membrane by alkali. The filter was then hybridized with 2.1 kb fragment of *cry1Ah* gene marked by α-^{32}P-dCTP as probe. The product of pUOAH digested by *Bam*H I and *Kpn* I served as an experimental positive control. Hybridization and membrane washing were carried out as described by Sambrook et al.[12].

1.7 Jin-Biao Assay to transformed maize plants

Take 0.2—0.4 g maize green leaf samples, grind it fully, add 4 mL ddH_2O, and allow the solids to settle in each tube for a few minutes. Put the Jin-Biao immune detection strip in 500 μL supernatant liquor uprightly, take out the strip after 5—10 min, and read the result. Then the strip will show two colorful lines to the plants expressing Cry proteins, one is the contrast line, and the other is the detection line. Negative results will only show a contrast line.

1.8 ELISA assay to transformed maize plants

ELISA analysis to transformed maize plants was performed according to the EnviroLogix QuantiPlate Kit handbook.

1.9 Insect bioassays

The Asia corn borers were provided by Institute of Plant Protection, Chinese Academy of Agricultural Sciences. When the T_2 plants reached 6 leaves to 8 leaves (V6—V8), 40—60 neonate larvae were introduced into the whorl of each plant for field insect resistant assay. Non-transformed plant was taken as the negative control. The assessment of insect-resistance adopted nine grading standard established by International coordination team of corn borers.

2 Results and analysis

2.1 Molecular analysis of T_0 transformed maize plants

(i) The expression of Bt insecticidal protein in T_0 transformants. On the basis of efficient regenerated system of maize (*Zea mays* L.) hybrid line Q31×Z3, 260 primary transformants were obtained. The expression of Bt insecticidal protein in a total of 260 T_0 plants was examined by Jin-Biao immune detection strips. 144 in 260 T_0 plants expressed the Bt insecticidal protein. No Bt insecticidal protein was observed in untransformed plant. 70 in 144 T_0 plants were pUUOAH transformants; 36 in 70 were reproductive; 74 in 144 T_0 plants were pUOAH transformants; 32 in 74 were reproductive. Before making use of the Jin-Biao strips, we carried out pre-experiment using purified Cry1Ah protein, and made sure that the least concentration of Cry1Ah protein for observation was 20 pg/μL (data not shown).

(ii) PCR analysis and Southern blot analysis to T_0 transformed maize plants. One hundred and forty-four T_0 transformants expressing Bt insecticidal protein were analyzed with PCR using *cry1Ah* gene specific primers F2 and R2. The expected 684 bp band was seen in each of the 144 transformants (Figure 2). No corresponding band was seen in untransformed control samples.

According to the results of Jin-Biao and PCR assay, 10 reproductive plants of each vector were selected for further analysis by Southern blot. Fifteen microgram

maize genome DNA was isolated from each young leaf tissues, digested by *Hin*d III, and hybridized with 2.1 kb fragment of *cry1Ah* gene. The product of pUOAH digested by *Bam*H I and *Kpn* I served as an experimental positive control. The positive control gave two bands, which suggested the incomplete digestion of the plasmid pUOAH .And a total of 20 plants gave excepted bands, which verified the integration of *cry1Ah* gene into the genome of maize transgenic plants from different cases (Figure 3).

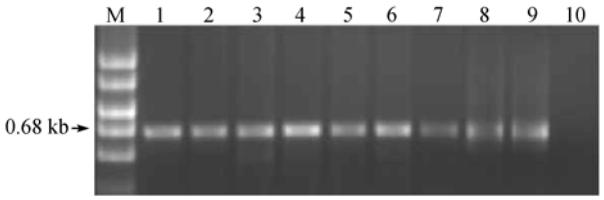

Figure 2 PCR analysis of T_0 plants regenerated from hygromycin-resistant calli. 1, plamid pUOAH with *cry1Ah* gene; 2—9, transgenic plants; 10, untransgenic plants. M, DL2000 Marker.

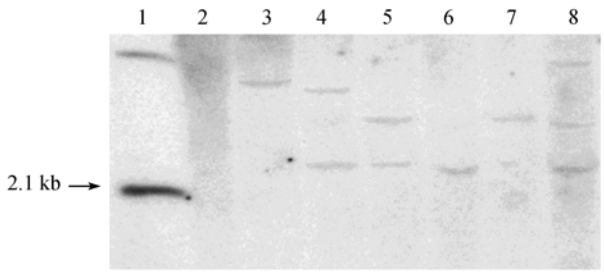

Figure 3 Southern blot analysis of T_0 plants regenerated from hygromycin resistant calli. 1, plamid pUOAH digested by *Bam*H I and *Kpn*I; 2, untransgenic plant; 3—8, transgenic plants.

2.2 Molecular analysis of T_1 transformed maize plants

(i) PCR analysis and Southern blot analysis to T_1 plants. After self-pollination, Southern blot-positive T_0 plants progenies were selected to seed in the field. 169 T_1 plants were obtained, and all of them were analyzed with PCR using *cry1Ah* gene specific primers F2 and R2. The results showed that a total of 20 lines showed segregation .The expected 684 bp band was seen in 90 T_1 plants, and no corresponding band was seen in untransformed control samples. PCR-positive plants were further analyzed by Southern blot. 50 PCR-positive plants gave excepted bands, which was the same as their primary transformants (Figure 4). Twenty-four plants in 50 were pUUOAH progenies, and the other 26 plants were pUOAH progenies. These results indicated that the *cry1Ah* gene had been integrated into maize genome and

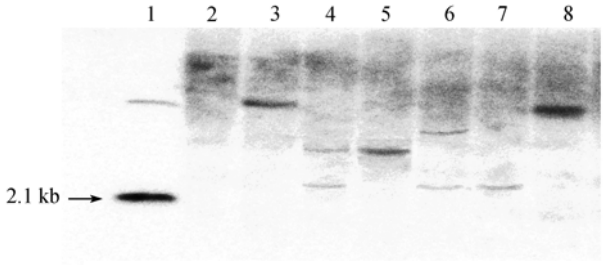

Figure 4 Southern blot analysis of T_1 plants regenerated from hygromycin resistant calli. 1, plamid pUOAH digested by *Bam*H I and *Kpn*I; 2, untransgenic plant; 3—8, transgenic plants.

inherited to the next generation stably.

(ii) ELISA analysis to T_1 plants. When the T_1 plants reached V10, two Southern blot-positive plants from each T_1 line were selected for ELISA assay. A total of 40 plants all expressed Bt insecticidal proteins, but the levels of Cry1Ah protein varied greatly among independent plants, ranging from 5.12 to 23.8 μg/g FW (Fresh Weight). The expression of Cry1Ah in most pUUOAH progenies was higher than that of pUOAH progenies. The average expression of Cry1Ah in 20 pUOAH plants was 11.8 μg/g FW, and 14.07 μg/g FW in 20 pUUOAH plants which was 19.2% higher than that of the intronless construct (pUOAH). The results of ELISA assay showed that the *ubi1* intron could efficiently enhance the expression of *cry1Ah* in transgenic maize (Table 1).

Table 1 The expression of Cry1Ah in transgenic maize leaves (μg/g FW)

		pUOAH	pUUOAH
T_1 transgenic maize	The highest expression of Cry1Ah	23.80	19.79
	The lowest expression of Cry1Ah	7.87	5.12
	The average expression of Cry1Ah	14.07	11.80
T_2 transgenic maize	The highest expression of Cry1Ah	43.38	17.08
	The lowest expression of Cry1Ah	4.31	4.25
	The average expression of Cry1Ah	13.98	11.40

2.3 ELISA assay and bioassay to T_2 plants

(i) ELISA assay to T_2 plants. After self-pollination, Southern blot and ELISA positive progenies were selected to seed in the field. Thirty plants from each vector were selected randomly to analyze the Bt insecticidal protein expression. A total of 60 plants all expressed Bt insecticidal proteins, but the levels of Cry1Ah varied greatly among independent plants, ranging from 4.25 to 43.38 μg/g FW. The average expression of Cry1Ah in 30 pUOAH plants was 11.4 μg/g FW, and 13.98 μg/g FW in 20 pUUOAH plants, which was 22.6% higher than

that of the intronless construct (pUOAH)(Table1).The results of ELISA assay showed that the *ubi1* intron-mediated enhancement had inherited to the next generation stably.

(ii) Bioassay to T_2 plants. When the T_2 plants reached V6—V8, we carried out insect resistant bioassay. Significant tissue damages were seen in untransformed plants, and there were many large wormholes in the leaves; nevertheless, in each transgenic line, most transgenic plants showed resistance to the corn borers, although there are few negative plants (Figure 5). We obtained 125 high resistant plants, 75 of 125 were pUUOAH transformants, and the other 50 were pUOAH transformants. The number of high resistant plants of each vector is shown in Table 2. It showed that the resistance of pUUOAH progenies is higher than that of pUOAH, and the number of resistant plants in pUUOAH progenies is larger than that of pUOAH progenies.

Figure 5 Bioassay to T_2 plants. (a) Untransgenic plant; (b) transgenic plant.

Table 2 The statistics of T_2 plants resistant to the corn borers

	pUUOAH	pUOAH
Grade 1	29	20
Grade 2	29	14
Grade 3	17	16
Total	75	50

3 Discussion

It has been more than 10 years since transgenic maize expressing insecticidal proteins was first commercialized in USA, but most transgenic cases still take *cry1Ab* or *cry1Ac* gene as materials in the research of IR maize. The exploitation of novel genes as materials for IR maize can efficiently overcome the issue of monotonous Bt genes, delay levels of resistance risk evolved by the insect species. The *cry1Ah* gene has been patented, which exhibited higher toxicity to lepidopteron insects than any other *cry* genes. The *cry1Ah* transgenic maize acquired in this study had high resistance to the Asian corn borers, which demonstrated the promising prospect of the utilization of *cry1Ah*. The utilization of *cry1Ah* in IR maize is helpful to the exploitation and industrialization of IR maize in China and lay basis for the commercialization of Chinese IR maize.

Though the *cry1Ah* transgenic maize has a promising future, appropriate resistant management (IRM) tactics must be adopted, because the threat of potential resistance of insect populations still exists. Ferre et al.[13] found that *Plutella xylostella* and *Trichoplusia ni* had evolved significant levels of resistance in the laboratory and the field, and laboratory selection experiments had shown the high potential of other species to evolve resistance against Bt. The first generation of resistant management tactics in the world is high-dose/refuge strategy, but the required size of refuge in this strategy is large (20%—50%). It is difficult to popularize in developing countries (such as China and India) predominant with small farmers. The second generation IRM technology is stacking two existing toxins in one variety[14], such as Bollgard II Bt cotton expressing two Bt genes(*cry1Ac* and *cry2Ab)* and Bt maize containing *cry1Ab* and *cry1F* genes, which appears to be the best way to delay resistance of insect populations until now. The toxic fragment of Cry1Ah was 72%, 82% similar to that of Cry1Ab and Cry1Ac respectively. Bioassay has showed that there was partial cross-resistance between them (data not shown). Another Bt gene *cry1Ie* has also been patented, which exhibited moderate toxicity to lepidopteron insects, and had no cross-resistance with *cry1Ah*. The transformation of *cry1Ie* transgenic maize is in process. In the next step, we would try to get varieties containing two Bt genes by traditional hybridization between the *cry1Ah* inbred line and *cry1Ie* inbred line. So the *cry1Ah* transgenic maize acquired in this paper is valuable to the cultivation of new Bt varieties containing two Bt toxins.

The ELISA assay to T_1 and T_2 generation plants showed that the expression of *cry1Ah* gene in the construct containing the *ubi1* intron (pUUOAH) was 20% higher than that of the intronless construct (pUOAH). Except the *ubi1* intron, all elements in pUUOAH vector are identical to that of pUOAH. The plant materials used for microprojectile bombardment are entirely the same,

so the enhancement of Cry1Ah is the results of *ubi1* intron- mediated enhancement. Vain et al.[15] reported that the *ubi1* intron provided 26-fold enhancement of gene expression of maize in transient system when the report gene *uidA* was driven by CaMV35S promoter. The maize *ubi1* intron used in this paper was the same as that of Vain's, but the promoter used here was maize Ubiquitin promoter. Some studies pointed out the degree of intron-mediated enhancement might be inversely proportional to the basal activity of the promoter employed[16]. Furthermore, maize Ubiquitin promoter used here has contained the *ubi1* intron of maize (to elevate the expression of genes, the maize ubiquitin promoter contained the *ubi1* intron is widely used in plant genetic transformation). In fact, the *ubi1* intron used here is equal to the introduction of two tandem introns in pUUOAH, one intron in pUOAH. Though the levels elevated is lower than the CaMV35s promoter employed, we can conclude that the tandem of introns can still enhance the expression foreign genes. Tandem introns mediated enhancement is still needed in future exploitation.

It was confirmed that more than 40 species of bacterium have the abilities of natural transformation[17]. For example, *Pseudomonas* could integrate free DNA in the soil into their genome by natural transformation. So when the transgenic plants are released to the environment, foreign genes can enter into the transfer chain of bacteria by transformation, recombination and transduction, which might be adverse to the microorganism in the soil. Prokaryotes have no intron splicing system , so if we put the intron into the coding sequence of foreign genes, not only the expression of foreign genes can be enhanced ,but also the foreign gene will not allowed to be transformed to microorganism. This is another notable function of introns besides IME. We will continue to exploit the mechanism of intron-mediated enhancement in the coding sequence of genes as well as the function of introns on the biosafety.

1 James C. Global Status of Commercialized Biotech/GM Crops: 2006. ISAAA Brief No. 35. ISAAA: Ithaca, 2006
2 James C. Global Status of Commercialized Biotech/GM Crops: 2007. ISAAA Brief No. 37. ISAAA: Ithaca, 2007
3 Shelton A M, Zhao J Z, Roush R T. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annu Rev Entomol, 2002, 47: 845－881
4 Castle L A, Wu G S, McElroy D. Agricultural input traits: past, present and future. Curr Opin Biotech, 2006, 17:105－112
5 Xue J, Liang G, Crickmore N, et al. Cloning and characterization of a novel Cry1A toxin from *Bacillus thuringiensis* with high toxicity to the Asian corn borer and other lepidopteran insects. FEMS Microbiol Lett, 2008, 280(1): 95－101
6 Callis J, Fromm M, Walbot V. Introns increase gene expression in cultured maize cells. Genes Dev, 1987, 1: 1183－1200
7 Jeong Y M, Mun J H, Kim H, et al. An upstream region in the first intron of petunia actin depolymerizing factor 1 affects tissue-specific expression in transgenic *Arabidopsis*. Plant J, 2007, 50: 230－239
8 Maniatis T, Reed R. An extensive network of coupling among gene expression machines. Nature, 2002, 416: 499－506
9 Proudfoot N J, Furger A, Dye M J. Integrating mRNA processing with transcription. Cell, 2002, 108: 501－512
10 Zhang X J, Liu J Q, Zhao Q, et al. Transfer of high Lysine gene into maize by microprojectile bombardment and detection of transgenic plants (in Chinese). J Agricult Biotechnol, 1999, 7: 363－367
11 Soyle J J, Doyle J L, Hortorium B H. Isolation of plant DNA from fresh tissue. Focus, 1996, 12(1): 13
12 Sambrook J, Russell D. Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2001
13 Ferre J, Van R J. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol, 2002, 47: 501－533
14 Bates S L , Zhao J Z, Roush R T, et al. Insect resistance management in GMcrops: Past, present and future. Nat Biotechnol, 2005, 23(1): 57－62
15 Vain P, Finer K R, Engler D E, et al. Intron-mediated enhancement of gene expression in maize and bluegrass. Plant Cell Rep, 1996, 15: 489－494
16 Lorkovi Z J, Wieczorek Kirk D A, Lambermon M H L, et al. Pre-mRNA splicing in higher plants. Trends Plant Sci, 2000, 5(4): 160－167
17 Vries J, Herzfeld T, Wackernagel W. Transfer of plastid DNA from tobacco to the soil bacterium Acinetobactersp by natural transformation. Mol Microbiol, 2004, 53(1): 323－334