Functional Analysis of Transgenic *Cry1Iem* Maize

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Abstract

The genetic transformation of maize callus was induced by *Agrobacterium tumefaciens*. The *Cry1Iem* was transferred into callus of inbred line H99 maize, and the transgenic plants were detected by herbicide screening and PCR. A further Southern blotting experiment, real- time quantitative PCR and indoor/outdoor insect resistance identification test were carried out on T2 transgenic plants. The results of Southern blotting of transgenic plants showed that *Cry1Iem* had been successfully integrated into the maize genome. Real-time quantitative PCR showed that *Cry1Iem* was successfully expressed in transgenic plants. The insect resistance test showed that the resistance of the transgenic plants was significantly improved.

1 Introduction

According to the statistics from UN Food and Agriculture Organization, Chinese corn production has been increasing since 2003. In fact, China is the second largest corn producer in the world after the United States. However, the yield of Chinese corn is relatively low. High production costs and poor quality result in weak competitiveness. Every year, a large number of international low-cost corn flow to China and have a serious impact on our domestic stockpile, which in turn lead to a drop in prices. Farmers are demotivated by small profits from corn production and are no longer reluctant to plant it. The vicious circle causes a severe depression in Chinese domestic corn production chain. Therefore, it is of great significance to improve the quality and the yield of Chinese corn.

Ostrinia nubilalis is a kind of Lepidoptera Crambidae insects, which is the most important insect pest affecting corn yield and corn quality in China's corn production. Borers harm to corn by damage its different parts throughout growth (e.g. damage young stems and leaves at seedling stage, damage corn buds during grain filling stage, damage flowering tassel and filaments in flowering phase, ect.) (Al-Eryan et al. 2020). The damage could reduce photosynthetic capacity, damage water transport system and disturb pollination of maize plants, which directly led to the reduction in corn yield and the decline of grain quality. Insect resistant transgenic maize which has many unique advantages over insecticide control protects against corn borers fundamentally by expressing insecticidal proteins. At present, Bt gene is the most widely used insect-resistant gene. It uses the expressing insecticidal proteins such as *Cry1Ab*, *Cry1Ac* and *Cry1Iem* (Nguyen et al. 2007; JD Ríos-Díez et al. 2017; Wang et al. 2017). A transgenic *Cry1Ab* gene was produced by the Monsanto Company in the United States .It has significant resistance to the Lepidoptera corn borers and has no adverse effect on other agronomic traits of corn. Also, it can effectively prevent and control the damage of corn borers. Research on insect-resistant transgenic maize in China started late. There is still no transgenic insect-resistant maize with independent intellectual property rights available commercially. Zhang, Y.et al.(Zhang et al. 2016) isolated a new type of Bt insecticidal crystal protein gene *Cry1Ie1* from Bacillus thuringiensis, which showed high insecticidal activity against corn borers. Yun-Jun LIU. et al.(Yun-Jun et al. 2004) transformed this gene and named it *Cry1Iem*. This new gene not only retained the high insecticidal activity of *Cry1Ie1* against Ostrinia furnacalis, but also significantly increased its expression in plants. In this study, it is proposed to

introduce the gene *Cry1Iem* into *Agrobacterium tumefaciens*-mediated method to obtain highly resistant to Ostrinia furnacalis transgenic plants and to provide new germplasm for the development of new resistant varieties to insect-resistant transgenic maize.

2 Materials And Methods

2.1 Test Materials

Plant Materials: Maize (*Zea mays* L.) inbred line H99;Experimental strains: *Ecoli.* DH5α, *Agrobacterium* EHA105; Plant expression vectors: pCAMBIA3300-cry1Iem-Bar Test Insects: Asian corn borer larvae provided by Jilin Agricultural University Institute of Modernization.

2.2 Major reagents

Plasmid extraction kit was purchased from Wigras, the plant genomic DNA extraction kit was purchased from Kangwei, and the Southern blotting kit was purchased from Roche. The reverse transcription kit was purchased from Fermentas company and the plant protein extraction kit was purchased from Bioengineering company.

2.3 Agrobacterium Tumefaciens Mediated Transformation of Maize

Callus pCAMBIA3300-cry1Iem-Bar (Tab.1) was transformed into *Agrobacterium tumefaciens* EHA105 to make the engineered bacteria. The engineered bacteria were expanded propagation to prepare the infected bacterium liquid and the callus blocks induced by the immature embryos of H99 were infected. After infection, resistance calluses were screened and differentiate into seedlings (Fig. 1).

Table 1
Primer sequence

Name	Primer sequence
Iem-F	GCCTGTCTAGTAACGCTAAA
Iem-R	GGCCTTTTCGAAATCGTATT
Bar-F	TCAAATCTCGGTGACGGGC
Bar-R	ATGAGCCCAGAACGACGC
Actin-F	ATCTTGACTGAGCGTGGTTATTCC
Actin-R	GCTGGTCCTGGCTGTCTCC

2.4 Detection of progeny of transgenic maize plants

2.4.1 PCR detection of transgenic plants

The regenerated plants were screened for herbicide phosphinothricin to extract DNA from resistant seedling leaves. The DNA of the untransformed plants was used as a negative control, and the expression vector plasmid was used as a positive control. The *Cry1Iem* gene and Bar genes were amplified respectively from the original expression vector (reaction conditions: 94℃ for 5 min, 94℃ for 30 s, annealing temperature 45℃, 55℃, annealing time is 30 s, extension at 72℃ for 30 s, extension at 72℃ for 8 min, 30 cycles. The amplified products were separated by 1% agarose gel electrophoresis. DNA extraction methods refer to Kang century company plant genome extract kit instruction.

2.4.2 Southern Hybridization Test of Transgenic Plants

The CTAB method (Matthes et al. 2020) was used to extract a large number of PCR-positive genomes and non-transformed plants. pCAMBIA3300-cry1Iem-Bar was used as a positive control. Hind was used to digest the genomic plasmid and plasmid overnight, and then was separated by 0.8% agarose gel electrophoresis, The purified *Cry1Iem* gene was used as a template to prepare the probe. Then, the probe was labeled with DIG by a random primer method. The specific operation was performed according to the method described by Roche's DIG High Prime DNA Labeling and Detection Starter Kit I. The preparation of hybrids and reagents reference Jiao, P.et al (Jiao et al. 2021).

2.4.3 RT-PCR Test of Transgenic Plants

Whorl leaves of the total RNA tested positive, which were extracted and reverse transcribed into cDNA for PCR amplification. Specific primers Cry1Iemr-F and Cry1Iemr-R were used to amplify the *Cry1Iem* gene. The Actin gene was used as a reference gene, Action-R and Action-F are also primers. The reaction conditions were denaturation at 94℃for 5 min, denaturation at 94℃ for 30 s, annealing temperature at 45℃, 50℃, annealing time at 30 s, extension at 72℃ for 30 s, extension at 72℃ for 8 min and 30 cycles respectively.

2.5 Transgenic Plants Insect Resistance Identification

2.5.1 Transgenic Plants indoor Insect Resistance Identification

In the selective antifeedant experiment, after disinfection of fresh and flat Bt maize and non-Bt control maize leaves, cut into 2 cm wide and 3 cm long segments, leaf areas of 4 leaf segments, Bt corn and conventional corn segments were arranged in a Petri dish with a diameter of 13 cm (with filter paper inside and a little distilled water dripping to keep moisture), and then were measured by grid paper after starvation for 4 h and 2 instar larvae of Ostrinia furnacalis for 1, 8, 16, 24, 36 and 48 h, respectively. Non-selective antifeeding test: Leaf segments of single Bt corn and non-Bt control corn were put into each dish, and other iso-selective antifeeding experiments were conducted, and the antifeeding rate was calculated according to Equation 2.

Equation1: Select antifeedant rate (%) = CK feeding area - processing feeding area \ CK feeding area + processing feeding area × 100.

Equation2: Non-selective antifeedant rate (%) = CK feeding area - processing feeding area \ CK feeding area × 100.

2.5.2 Identification of insect resistance in heart-leaf stage of transgenic plants

T_2 generation transgenic seeds and H99 inbred line seeds (control) were planted in the field according to their lines and randomly divided into 3 replicates with 20 plants per row and 25 cm plant spacing. The transgenic population was screened. Five positive plants were selected from each line to carry out the experiment of insect-inoculation (note that insecticides should not be sprayed during the whole growth period) and three repeated experiments were carried out in repeated plots. At 6-8 leaf stage, that newly hatch larvae of corn borer (Ostrinia furnacalis) were inoculate into the corncob leaves at high morning humidity, 30 larvae per plant were inoculated into the corncob leaves, and the level of leaf-feeding was investigated after 14 days. According to the size and number of wormholes on the damaged leaves, the stage of heart and leaf can be divided into damage grades, which is called leaf-eating grade. At present, most of the international standards for grading corn borer have been adopted (Tab. 1). The leaf-eating grade was investigated one by one, and the average value of each plant was used as the leaf-eating grade of the identified strain, and the resistance grade was determined according to the evaluation criteria in Tab. 1. The borer hole was investigated before harvest, and the borer hole was 2.5 cm in diameter. The average value of three repeated experiments was taken as the average value of the resistance to insect pests.

Average leaf level = Σ (food leaf level × number of plants at this level) / total number of plants investigated.

2.5.3 Identification of insect resistance in the silking stage of transgenic plants

When the plant grows to the silking stage (the filaments of the plant's ears protrude from the loquat leaves about 2 cm), the corn borer larvae (blackheads) are attached to the top of the corn silk in the early morning when the humidity is high, and each plant receives 30 insects. Head, the experimental design is the same as above, and the damage of the ear is investigated 14 days after the insect is collected. For the identification criteria of insect resistance, refer to the criteria for the classification of Asian corn endangerment and the evaluation criteria for the resistance of maize ears to Asian corn borer at the ear of corn specified by the Ministry of Agriculture. Statistics on the damage of plant, The mean of the three replicates was used as the basis for evaluating the insect resistance grade of the strain.

3 Results And Analysis

3.1 Transgenic Plant Regeneration

Agrobacterium-mediated method of callus infection obtained 40 seedlings after a series of regeneration processes (e.g.co-culture, screening, differentiation, rooting, etc.). The seedlings were transplanted and the herbicide screening of 3-leaf stage seedlings obtained 15 herbicide-resistant plants. The obtained resistant plants were transplanted into the soil and cultivated in the greenhouse for molecular detection.

3.2 PCR detection results of maize transgenic plants

Genomic DNA was extracted from the leaves of herbicide-tolerant plants. The non-transformed plant was used as a negative control and the pCAMBIA3300-cry1Iem-Bar plasmid was used as a positive control. The *Cry1Iem* and Bar genes were amplified by PCR (Fig. 2). Ten positive plants were obtained. The obvious *Cry1Iem* gene and Bar gene bands.

3.3 Transgenic Plants Southern Hybridization Test

Southern hybridization was carried out on the plants tested positive by PCR, and the test results are shown in Fig. 3. A total of four Southern-positive plants were obtained, The plants ere single-copy bands. The size of each hybridization band was significantly different, and negative control found no obvious hybridization bands. The results of Southern hybridization indicated that the *Cry1Iem* gene has been integrated randomly into the genome of maize in different parts.

3.4 Transgenic Plants RT-PCR Detection

In order to further study the transcript of exogenous gene in maize, total RNA was extracted from the seedling of Southern hybrid plants and transcribed into cDNA as a template. The *Cry1Iem* gene was amplified by PCR using the primers Cry1Iemr-F and Cry1Iemr-R. The internal standard gene (primer: Actin) was simultaneously amplified. The amplification products were detected by 1% agarose gel electrophoresis respectively. The result is shown in Fig. 4. Four transgenic positive plants can amplify two bands of *Cry1Iem* and Actin genes, while non-transgenic plants only Actin strip without *Cry1Iem* band. The results of RT-PCR indicated that the *Cry1Iem* gene has been successfully transcribed in transgenic plants.

3.5 Transgenic Plants Pnsect Resistance Identification

The results of selective and non-selective antifeeding experiments of Bt maize against Ostrinia furnacalis at the end of 2nd instar were consistent (Fig. 5). Bt maize had good antifeeding activity against Ostrinia furnacalis within 8, 16 and 24 hours after the experiment (antifeeding rate was over 50%), but the antifeeding effect of Bt maize gradually lost with time, and the antifeeding rate was almost zero at 48 hours. The results showed that the susceptibility of corn borer to Bt corn decreased with the increase of its age and the time of feeding on Bt corn. In addition, at the same time, the selective antifeeding rate was higher than the non-selective antifeeding rate, which indicated that the larvae of Ostrinia furnacalis ate less Bt maize leaves under the condition of selectivity, while the larvae of Ostrinia furnacalis ate more Bt maize leaves under the condition of non-selectivity, so that the non-selective antifeeding rate was lower.

3.6 Identification of insect resistance in transgenic plants

Experiments on the inoculation of transgenic and non-transgenic plants showed that the T_2 transgenic plants positive for PCR had strong resistance to Asian corn borer, and the plants grew well after two weeks of infestation (Fig. 6-A). The transgenic control group was affected by severe insect pest (Fig. 6-B). There were a large number of large wormholes in the heart and outer leaves, which were highly sensitive to corn borer, and the leaf level was greater than 7 grades. The leaf damage of the plant was investigated according to the plant's leaf damage status, and the average leaf damage level of the corn borer was determined. The average damage value of each plant was calculated in three repeated experiments. The plant resistance was evaluated according to the 9-level resistance evaluation standard, and the average value of the three repeated experiments was evaluated. The resistance level of this strain. Statistical analysis was carried out on the resistance of each strain see Tab. 2. The insect resistance of different transformation events was different. The strains L1, L4 and L5 reached high resistance level, and the plants were almost not eaten by Asian corn borer. There were only a few needle-like wormholes, which were significantly different from the control; the strains L2 and L7 showed insect resistance, and the heart leaves had individual wormholes, which were significantly different from the control; the strains showed L3 and L6. For the worms, the heart leaf and the outer leaves have a large number of large wormholes, and there is no significant difference compared with the control. However, a small number of plants in each insect-resistant strain showed a certain degree of susceptibility, again indicating the genetic separation of the offspring. These susceptibility and high-sensitivity events may be due to insufficient expression of *Cry1Iem* protein or environmental factors, resulting in the loss of insect resistance.

Table 2
The resistances to Asian corn borers of transgenic seeding

Line No.	Origin	Leaf-feeding level	Resistant grade
L1	T1-1-1	1.30±0.43a	(HR)
L2	T1-2-2	3.02±0.17a	(R)
L3	T1-2-3	7.02±2.17c	(S)
L4	T1-2-4	1.22±0.26a	(HR)
L5	T1-3-1	1.46±0.36a	(HR)
L6	T1-4-2	6.21±0.19c	(S)
L7	T1-5-1	3.12±0.31a	(R)
CK	H99	7.62±0.42	(S)

Note: The data in table are the means ± standard deviations of leaf-feeding levels from every line, a, b, c represents a significant difference from the non-transgenic inbred line respectively as at a $p<0.01$, b 0.01 p 0.05 and c 0.05 p, same as the following.

3.7 Identification of insect resistance in transgenic plants during silking

According to the larvae experiment of the transgenic plants in the T_2 generation of maize transgenic plants, most of the PCR-positive plants were resistant to Asian corn borer, and the ears were not affected by corn borer, while the corresponding H99 inbred lines were not. In the transgenic corn plants, the ears are severely damaged by corn borer.

According to the average of the degree of damage to the ear of each line, the resistance of the lines was evaluated. The results are shown in Tab. 3. The resistance of the transgenic lines to the corn borer was significantly different at the earing stage. It is the strongest and reaches the high level of resistance. Compared with the control, the difference is extremely significant. L2, L4 and L5 have better resistance and reach the level of insect resistance. Compared with the control, the difference is extremely significant; the resistance of the strain L7 is relatively weak. The level of medium resistance was reached, and the difference was extremely significant compared with the control; while L3 and L6 had no obvious insect resistance compared with the control, and showed the level of insect resistance. Compared with the identification results of seedling resistance, the insect resistance levels of each strain decreased, but the relative levels of insect resistance of each strain were basically the same. The analysis may be due to the decrease of the expression level of *Cry1Iem* gene in the ear stage. The insect-resistant effect decreased, or the objective damage situation referenced in the evaluation criteria for seedling and ear-breaking insect resistance was inconsistent.

Table 3
Survey result in the field under artificial infestation with Ostrinia furnacalis at silking

Line No.	Origin	Mean damage rating scales	Resistant grade
L1	T1-1-1	1.00±0.21a	(HR)
L2	T1-2-2	3.22±0.31a	(R)
L3	T1-2-3	7.67±0.42c	(S)
L4	T1-2-4	2.21±0.23a	(R)
L5	T1-3-1	2.01±0.69a	(R)
L6	T1-4-2	6.02±0.45c	(S)
L7	T1-5-1	4.55±0.35a	(MR)
CK	H99	6.75±0.44	(S)

Notes：The data in table are the means±standard deviations of ear damagerating scales from threerepeated experiments.

4 Discussion

Insect-resistant effects are fundamental to the application of insect-resistant transgenic maize (CM 2021). Numerous studies have shown that the Bt transgenic plants have the strongest resistance to insect pest when compared to other insect-resistant genes at the same level of expression (Wang et al. 2016). *Cry1* gene is an important gene in the development of transgenic corn (Kriebs et al. 2017). Moar,W. J. et al. (Moar et al. 2017) showed that the structure of Cry protein conferred strong insecticidal activity against Plutella xylostella and tobacco budworm. The first batch of commercialized transgenic maize such as Bt11, Bt176, MON810 used in insect resistance genes, was this kind of gene (H et al. 2018;Li et al. 2016;Jana et al. 2018). *Cry1* gene crystal protein expression in maize can control the corn seedling stage and ear period of harm effectively (Strydom et al. 2019).

Insect-resistant genetically modified corn is mostly used as a raw material for feedstuff. Therefore, the potential risk of insecticidal crystal protein of transgenic corn in the growth and health of livestock and the safety of livestock products become a public concerns in the society (Han et al. 2016). Many researchers conducted cryogenic feed safety of transgenic corn. The results showed that the transgenic corn had no negative impact on the growth and health of the livestock (Xu et al. 2018).

In this study, *Cry1Iem* gene was introduced into maize inbred line H99 maize. Four transgenic plants were obtained by PCR, herbicide screening, southern hybridization and RT-PCR. Transgenic plants were tested for their resistance to maize boresr. The resistance is significantly improved, which has potential application value. However the safety needs further verification.Tan, Yanhua.et al. (Tan et al. 2019). pointed out that the efficacy of transgenic maize may be affected by the regional environment. Therefore, this study will conduct multi-year and multi-point field trials in the late identification of insect resistance and environmental safety to validate accurately the anti-insect effect of the transgenic events obtained in this study under different environments.

5 Conclusion

The results of Southern blotting of transgenic plants showed that *Cry1Iem* had been successfully integrated into the maize genome. Real-time quantitative PCR showed that *Cry1Iem* was successfully expressed in transgenic plants. The insect resistance test showed that the resistance of the transgenic plants was significantly improved.

Declarations

Acknowledgments We thank SYG and YYM conceived research plans and designed experiments.

Author's contributions Li-Yuan Han and Shu-Yu Fan designed and performed the experiments. Xiao-Tong Wei,Pi-Wu Wang analyzed the data,Li-Yuan Han and Shu-Yu Fan wrote the paper, Jing Qu,Shu-Yan Guan and Yi-Yong Ma full guided the experiments and the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Figures

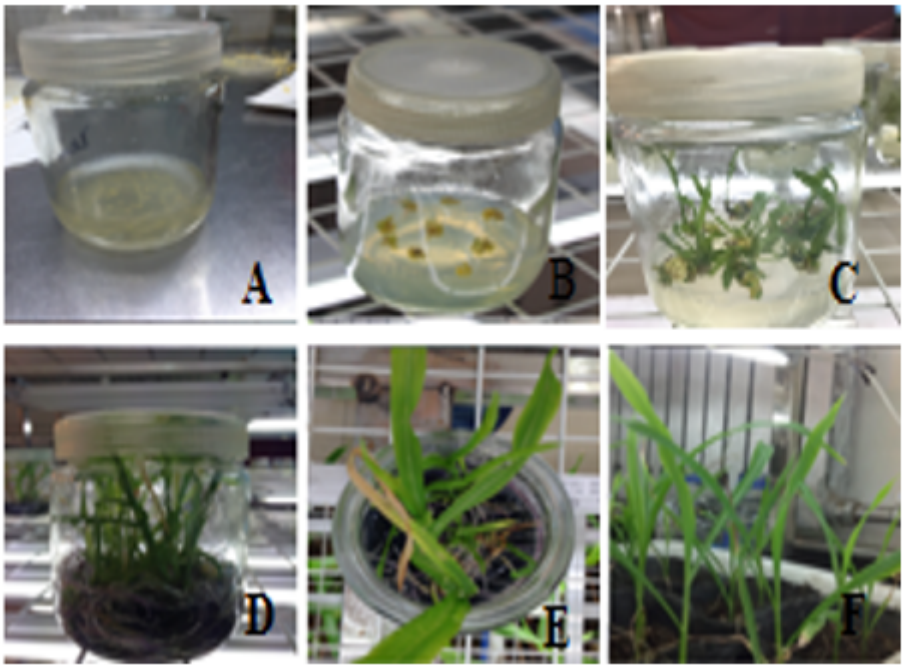

Figure 1

Agrobacterium mediated transformation of maize. A: Agrobacterium-mediated transformation B: Screening of resistant callus C: Differentiation culture D: Rooting culture E: Tempering seeding F: Transplanting seedlings

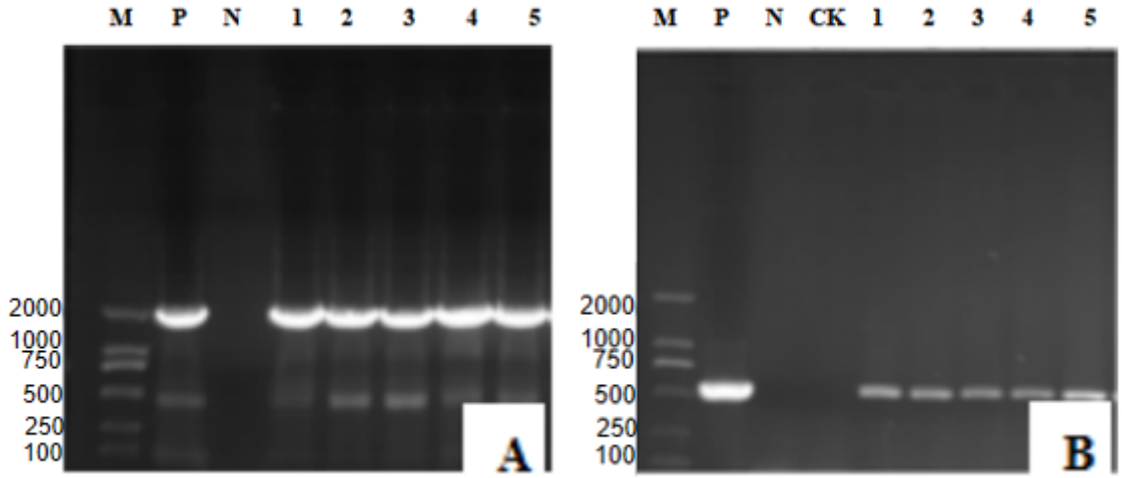

Figure 2

PCR detection of some transgenic maize. A：cry1Iem；B：Bar；M：DNA Marker DL 2000；P：positive control；N：negative control；CK：Non-transgenic plant；1-5: Transgenic plant.

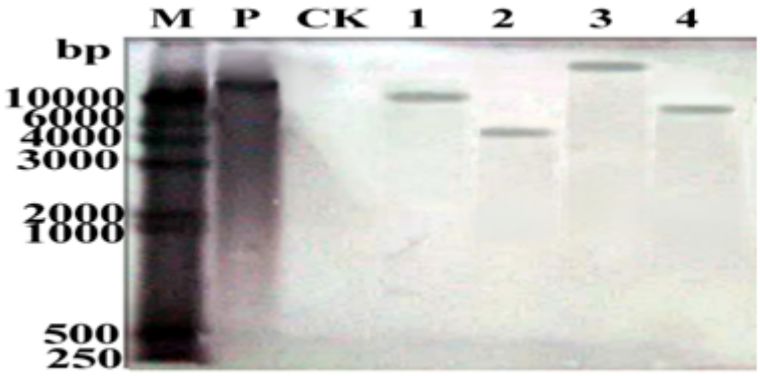

Figure 3

Southern blotting detection of transgenic plants with cry1Iem.M：DNA Marker DL10000；P：Positive control；CK：Non-transgenic plant；1-4: Transgenic plant.

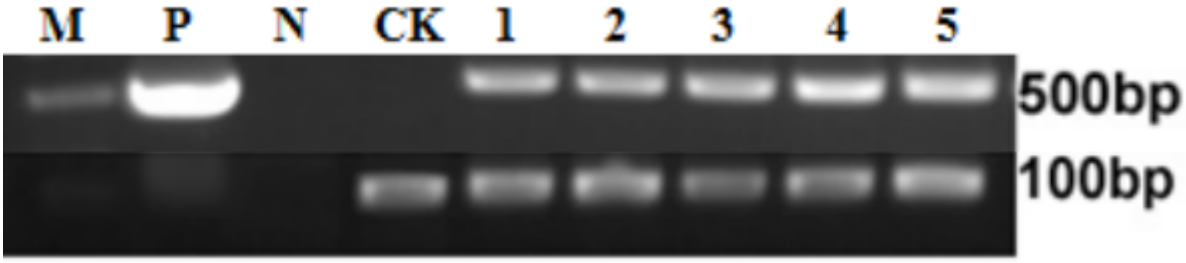

Figure 4

RT-PCR analysis of some transgenic plants.M：DNA Marker DL 2000；P：Positive control；N：Negative control；CK：Non-transgenic plant；1-5:Transgenic plant.

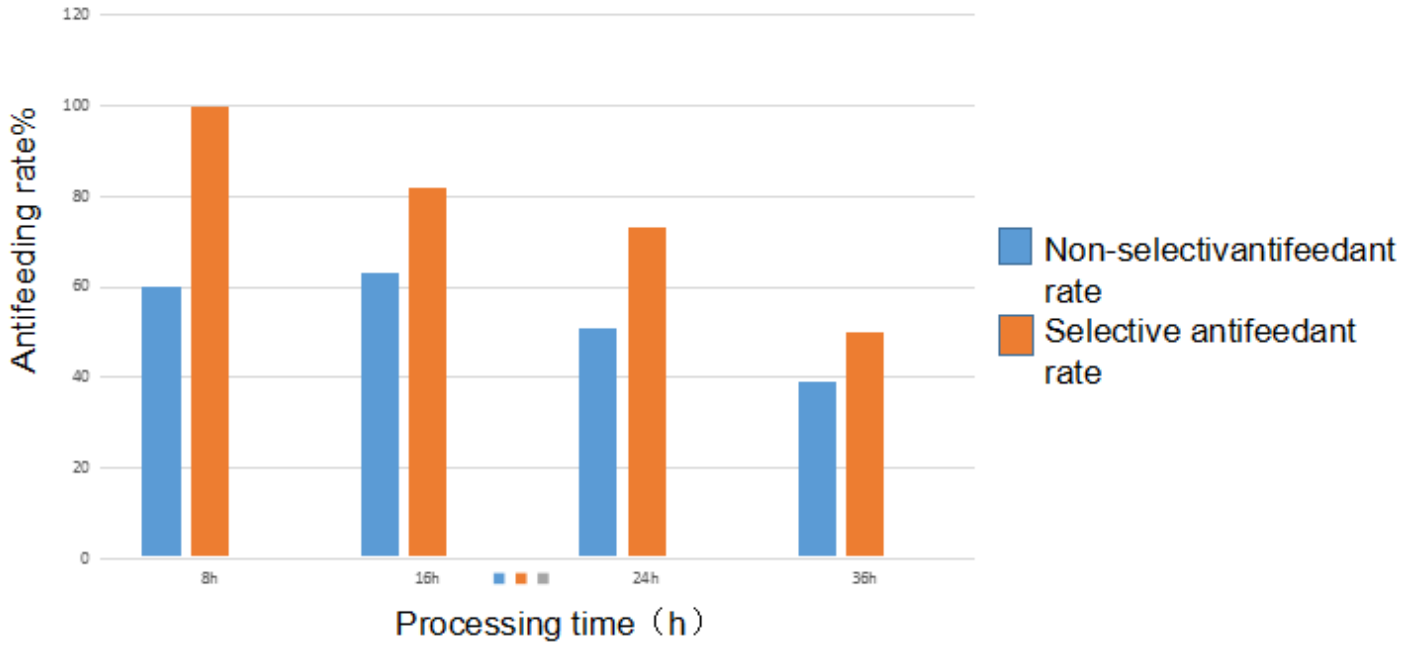

Figure 5

Antifeeding effect of Bt corn on corn borer larvae (2nd instar).

Figure 6

Evaluation of transgenic plants resistance to corn borers in the field. A：Transgenic plants with highly insect resistance；B：Non-transgenic control.