Cell Research (2001); 11(2):149-155

Agrobacterium tumefaciens-mediated transformation of rice with the spider insecticidal gene conferring resistance to leaffolder and striped stem borer

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ABSTRACT

Immature embryos of rice varieties "Xiushui11" and "Chunjiang 11" precultured for 4d were infected and transformed by Agrobacterium tumefaciens strain EHA101/pExT_7 (containing the spider insecticidal gene). The resistant calli were transferred onto the differentiation medium and plants were regenerated. The transformation frequency reached 56% ~ 72% measured as numbers of Geneticin (G418)- resistant calli produced and 36% ~ 60% measured as numbers of transgenic plants regenerated, respectively. PCR and Southern blot analysis of transgenic plants confirmed that the T-DNA had been integrated into the rice genome. Insect bioassays using T1 transgenic plants indicated that the mortality of the leaffolder (Cnaphalocrasis medinalis) after 7d of leaf feeding reached 38% ~ 61% and the corrected mortality of the striped stem borer (Chilo suppressalis) after 7d of leaf feeding reached 16% ~ 75%. The insect bioassay results demonstrated that the transgenic plants expressing the spider insecticidal protein conferred enhanced resistance to these pests.

Key words: *Rice, Agrobacterium tumefaciens, spider insecticidal gene, transgenic plant, Leaffolder, striped stem borer, insect bioassay.*

INTRODUCTION

Rice is one of the world's most important food crops. Although a decade ago rice was considered as one of the most recalcitrant crops in terms of genetic manipulation, it has recently emerged as the model cereal for the study of plant genomics, plant pathology, gene regulation and expression, metabolic pathway manipulation and the inheritance, organization, rearrangement and fate of transgenes[1],[2]. Remarkable progress has been made in the development of systems for Agrobacterium tumefaciences -mediated transformation in rice since 1994[3-8]. However, until now, only a few studies have been reported on A. tumefaciences-mediated transformation of rice using genes of agronomic importance to increase its resistance to pests[9],[10], and the numbers of plants obtained and the transformation frequencies in most of the studies mentioned above were unfortunately still very low (usually below 30%) from a breeder's point of view. Recently, more than 970 transgenic plants were produced with the modified SpI gene in two rice cultivars by using a modified Agrobacterium-based rice transformation procedure in our laboratory [7], PCR and Southern blot analysis indicated that the foreign genes had been integrated into the rice genome, and genetic analysis confirmed Mendelian segregation of the transgenes in subsequent generations. Insect feeding assays with the T1 plant tissues indicated that most of the transgenic plants tested were highly toxic to two major rice insects, striped stem borer

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Abbreviations: SpI=spider insecticidal gene; BAP=N6-benzyladene; NAA=a-naphthalene acetic acid; 2,4-D=2,4-dichlorophenoxy acetic acid; CH=casein, acid hydrolysate; AS=acetosyringone; Cef=cefotaxime; Kan=kanamycin; G-418=Geneticin; SSB= striped stem borer; LF=leaffolder.

Received Jan-31-2001 Revised April-28-2001 Accepted April-30-2001

and leaffolder, with near 38%-72% mortality within 7 d after infestation with few exception. Here we describe, for the first time, a method to efficiently transform rice by *A.* tumefaciences EHA101/pExT_7. We produced a large number of transformants of two rice cultivars and demonstrated stable integration, expression, and inheritance of transgenes.

MATERIALS AND METHODS

Plant materials and culture media

Japonica rice cultivars: Xiushui 11, Chunjiang 11 were used in transformation studies. Media used for tissue culture and transformation are listed in Tab 1. Immature seeds were dehusked, first sterilized with 75% ethanol for 2 min and then 6. 7% sodium hypochlorite for 15 min. The seeds were further washed 3 times with sterilized deionized water. The immature embryos were dissected aseptically and cultured on solid N_6[11] medium with 2 mg/L 2,4-D(designated N_6D_2). The cultures were incubated in the dark at 25±plusmnl;1 ºC for 4-5 d, the compact calli (1-2 mm in diameter) derived from the scutella were separated with scalpel and used for transformation.

A. tumefaciens strains, plasmid and culture

A. tumefaciens EHA101 harboring binary vector pExT_7 were used for the experiment. pExT_7 contained SpI (spider insect toxin) gene and nptII gene as a selectable marker within the T-DNA region. Each gene was under the control of an enhanced CaMV35S promoter (P35S). A. tumefaciens were grown overnight at 26ºC with shaking (210 rpm) in liquid YEP medium (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, pH 7.0) containing 50 mg/L kanamycin, 50 mg/L spectinomycin and 6 mg/L Tetracyclin with 100 μM acetosyringone, to mid-log phase (OD600=0.8-1.0). The culture was centrifuged at 3,000 rpm for 10 minutes and the pellet was resuspended in equal volume (usually 8 ~ 10 ml) of AAD1-AS medium (Tab 1) and used for co-cultivation.

Tab 1. Media used for tissue culture and transformation of rice

Medium	Composition
N6D2	N6[11], 500 mg/L casamino acids, 30 g/L sucrose, 2 mg/L 2,4-D,6.5 g/L agar, pH 5.8
N6D2-AS	N6D2 medium plus 100 μM acetosyringone
AAD1	AA[12], 500 mg/L casamino acids, 30 g/L sucrose, 1 mg/L 2,4-D, pH 5.6}
AAD1-AS	AAD1 medium plus 100 μM acetosyringone
N6CD2-CG	N_6CD_2 medium plus 300 mg/L cefotaxime and 25-50 mg/L G-418
RE1-CG	MS[13], 30 g/L sucrose, 10-20 g/L sorbitol, 1-2 g/L casamino acids, 2 mg/L 6-BA, 0.25 mg/L NAA, 250 mg/L cefotaxime and 50 mg/L G-418, 2.2 g/L gelrite, pH 5.8
RE2-G	MS medium plus 30 g/L sucrose, 10 g/L sorbitol, 1-2 g/L casamino acids, 2 mg/L 6-BA, 0.5 mg/L NAA, 50 mg/L G-418, 2.2 g/L gelrite, pH 5.8
MS0K	MS major salts, MS minor salts and MS vitamins, 20 g/L sucrose, 75 mg/L Kanamycin, 2.2 g/L gelrite, pH 5.8
1/2MSNK	Half-strength MS major salts, MS minor salts and MS vitamins, 0.5 mg/L NAA, 20 g/L sucrose, 75 mg/L kanamycin, 2.2 g/L gelrite, pH 5.8

Co-cultivation, selection and plant regeneration

The calli were soaked in bacterial suspension for 20 min, blotted dry with sterile filter paper to remove excess bacteria. Then the calli were transferred on a piece of filter paper placed on a co-culture medium (N_6D_2-AS). One ml of a liquid medium (AAD_1-AS) was dripped onto the surface of the filter paper. The plates were sealed with parafilm. Co-cultivation was carried out in the dark at 26ºC for 3 d. The calli labeled as control were not infected with Agrobacterium. After co-cultivation, the infected calli were washed 3 ~ 5 times with sterilized deionized water containing 500 mg/L cefotaxime to kill the Agrobacterium. The inoculated calli were first transferred to N_6D_2 medium containing 300 mg/L cefotaxime (N_6D_2C) for one week of callus growth without selection and then transferred to N_6D_2-CG medium (Tab 1). After selection for 3-4 w, resistant calli were transferred to RE1-CG medium for shoot development. The regenerated shoots were further transferred to RE2-G for full plantlet formation and then rooted on 1/2MSNK medium. After rooting, the transgenic plants were transferred to a glasshouse and grown to maturity.

PCR and Southern blot analysis of transgenic plants

1. DNA isolation Total genomic DNA was extracted from 0. 2-1.0 g young leaf tissues of transgenic plants and untransformed control according to CTAB method[14].

2. Polymerase chain reaction (PCR) analysis The putative transgenic (G-418 resistant) plants were analyzed by PCR for the presence of the nptII transgenes. One pair of specific primers were used to detect the npt II gene: forward primer (P1) 5'-AGAGGCGGCTATGACTGG-3' and reverse primer (P2) 5'-ATCGCCATGGGACGAGAT-3'. The PCR reactions were carried out in a total volume of 30 μl, comprising 50 ng of rice genomic DNA (1 μl), 20 pmol of each primer, 200 μM each dNTP, 0.5 units Taq DNA polymerase and 3 μl 10×PCR buffer. DNA was denatured at 94ºC for 5 min followed by 35amplification cycles (94ºC for 45s, 62ºC for 1 min, 72ºC for 1 min) and 10 min at 72ºC.

3. Southern blot analysis Total genomic DNA was isolated from 3-8 g fresh weight leaf material according to the manufacturer's instructions[15]. A 10- μg aliquot of DNA was digested overnight at 37ºC with restriction enzyme XhoI. The digested DNA was fractionated in a 0.7% agarose gel, transferred to a Hybond^{TM}- N^+ nylon membrane (Amersham). The plasmid pExT_7 was digested by XhoI and 2.0 kb fragment containing the spider insecticidal gene was used as probe labeled with α-^{32}P-dCTP (using Random Primer Label System). Hybridization and detection were performed according to Sambrook et al [15].

Inheritance of the marker gene

The selfed seeds (T_1 generation) of the transformants were sown in solidified MSoK medium with 75 mg/L kanamycin and cultured at 25±1°C. Kanamycin-resistance was scored 7-10 d after sowing. The segregation of the nptII gene in T_1 transgenic plants were determined.

Insect bioassays of transgenic plants

Insecticidal activity of the transgenic plants towards two major rice insects SSB (striped stem borer) and LF (leaffolder) was assayed similar to the described methods [8],[9]. The T0 and T1 generation of the transgenic rice plants that had been selected for Kanamycin-resistance and showed positive in South-

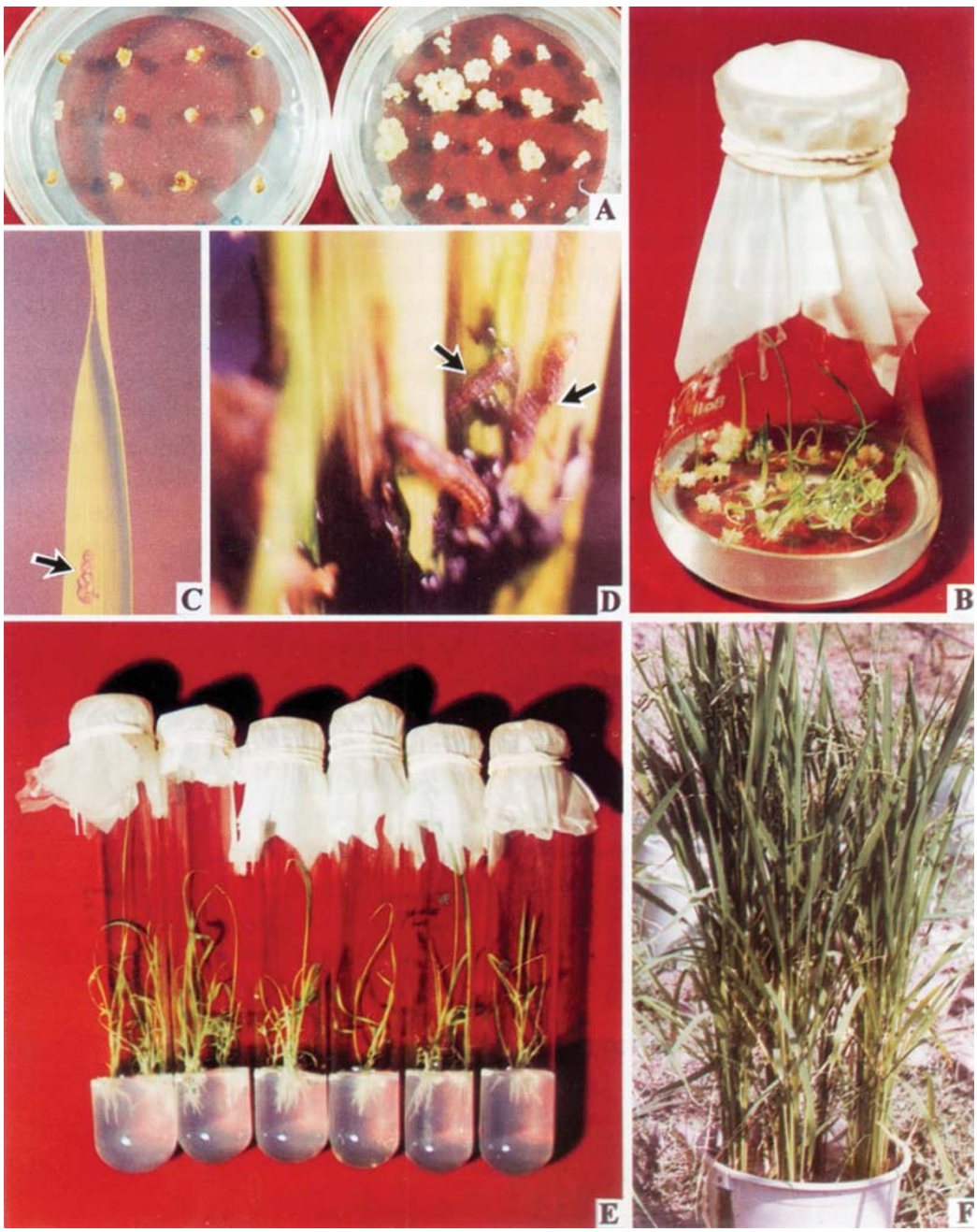

Fig 1. Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium tumefaciens EHA101/pExT$_7$
(A) Geneticin (G418)-resistant calli (right) after 2 w of selection, left: untransformed control calli; **(B)** Regenerated resistant shoots on RE-2G medium; **(C)** Dead leaffolder larvae 5d after leaf feeding on transgenic rice (arrow indicated); **(D)** Dead striped stem borer larvae 7 d after leaf feeding on transgenic rice (arrow indicated); **(F)** Transgenic rice plants after 30 d of leaf feeding with striped stem borer grew well and produce normal seeds.

ern blot analysis were used to perform insect-bioassays. Each of the tested plants were infected with 15 ~ 30 second-instar larvae of SSB or LF for 7 d, and larval damage and leaf area consumption or the mortality of insects were monitored. For SSB-resistance test, only larvae found inside the stems were recorded. The larvae of SSB were obtained from the Institute of Entomology, CAS and the larvae of LF were collected from the rice field of Xinzhuang region, Shanghai. Nontransgenic plants, which had no kanamycin-resistance and shown negative in Southern blot analysis, were used as negative controls.

RESULTS AND DISCUSSION

Co-cultivation, selection and plant regeneration

Various factors including different explant types, A. tumefaciens cell density for inoculation, time period of inoculation and co-culture, co-culture medium, and induction agents in the inoculation and co-culture media were found to affect the efficiency of Agrobacterium-mediated transformation. Optimization of the condition of co-cultivation was a critical importance[3]. Embryogenic callus derived from immature embryos 4 d after culturing on N_6D_2 medium were inoculated and co-cultured with A. tumefaciens EHA101/pExT_7. After co-cultivation, the infected calli were transferred to N_6D_2C medium (N_6D_2 medium plus 300 mg/L cefotaxime) for one week, and then transferred to N6D2-CG medium (Tab 1). After selection for 3-4 w, resistant calli (Fig 1-A) were transferred to RE1-CG medium for shoot development (Fig 1-B). The regenerated shoots were further transferred to RE2-G for full plantlet formation and then rooted on 1/2MSNK medium (Fig 1-E). Efficiency of Agrobacterium-mediated transformation of two rice cultivars was listed in Tab 2. The results indicated that, the transformation efficiency of Xiushui 11 reached 71.6% (resistant callus production) and 60.3% (transgenic plant regeneration), which was much higher than that of Chunjiang 11 (56.3% and 36.2%, respectively). It showed that proper genotypes selection is a critical factor in achieving efficiently transformation of rice. This conclusion is in agreement with that of Aldemita[4] and Zhang [5]. The selectable marker present in the T-DNA of the binary vector is also an important factor. The nptII gene present in EHA101 required a long exposure to high concentration of G418 in order to obtain a high efficiency of stably transformed calli. Our results showed that G418 is a good selectable marker, and it allowed good discrimination between transformed and nontransformed tissue. High frequencies of resistant callus production and plantlet regeneration were obtained on G418-containig medium (Tab 2). This conclusion is in contrast to that of Raineri[16], Chan[17] and He[18]. In our study, the high efficiency of obtaining transgenic plants was obtained in immature embryos infected with EHA101/pExT_7 preinduced with AS followed by co-cultivation in the presence of AS. In contrast, no transgenic plants obtained without AS treatment (data not shown). It showed that acetosyringone can indeed induce vir gene expression and extend the host range of some A. tumefaciens strains.

Characterization of the transgenic plants

Transgenic plants were grown in a growth chamber and evaluated for morphology and fertility. More than 110 events were established in soil and examined (data not shown). The majority (about 95%) of the transformed plants were fertile and produced as many seeds as the seed-derived control plants. We analyzed all of the independently derived transgenic plants by PCR for the presence of the nptII transgenes. As shown in Fig 2, we found that the 7 transformed plants randomly

Tab 2. Efficiency of Agrobacterium-mediated transformation in two rice cultivars

Rice cultivar	Experiment No.	Number of co-cultivated calli (A)	No. of G418^r calli (B)	No. of callus producing plants (C)	No. of G-418^r plants	Transformation efficiency (%) B/A	C/A
Xiushui 11	1	108	74	65	229	68.5	60.2
	2	135	98	83	274	72.6	61.5
	3	92	68	54	117	73.9	58.7
	Total	335	240	202	620	71.6[a]	60.3[a]
Chunjiang 11	1	68	35	29	86	51.5	42.6
	2	93	46	30	112	49.5	32.3
	3	118	76	42	153	64.4	35.6
	Total	279	157	101	351	56.3[b]	36.2[b]

[a,b] Average number of transformation efficiency.

tested contained the 521bp fragment of *nptII* gene, and the size of the amplified fragment is in coincidence with that of the positive control. It indicated that the foreign nptII gene had been integrated into the rice genome.

The status of the transgenic plants were characterized by Southern blot analysis in detail, and the copy number of the SpI gene in transgenic plants were estimated. DNA was extracted from leaf tissue of 45 T_0 plants derived from independent events, and digested with XhoI and hybridized with a probe (2.0 kb XhoI-digested fragment of $pExT_7$ containing the spider insecticidal gene) labeled with α-^{32}P-dCTP. We analyzed 9 independently derived T_0 transgenic plants by Southern blot (Fig 3). Unique and complex hybridization patterns were revealed, indicating that these plants were indeed originated from independent events. Because XhoI cuts only once in SpI probe, the number of hybridization bands is in agreement with the copy number of T-DNA integrated into transgenic plants. Among the 9 transgenic plants tested, all of them showed positive hybridization

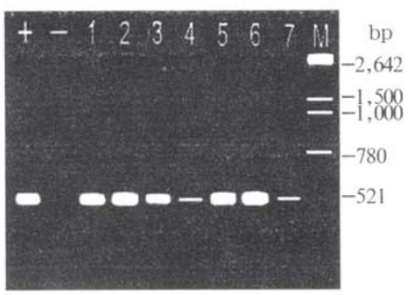

Fig 2. PCR assay of nptII gene in transgenic rice plants M: Molecular weight marker; +: Positive control (521bp fragment from pGIH; -: An untransformed control plant; 1-7: Transgenic plants.

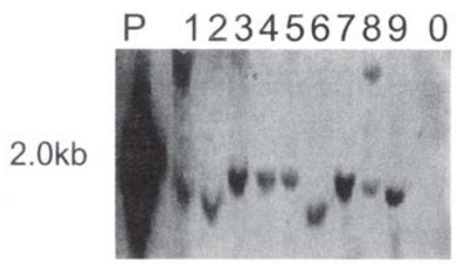

Fig 3. Southern blot analysis of transgenic rice plants P: Positive control (2.0kb XhoI fragment from $pExT_7$); O: An untransformed control plant; 1-9: Transgenic plants.

bands of SpI gene. Most of the hybridization bands were between 1.8 and 4.0 kb. Most of the transgenic plants carried one copy of the SpI gene, with only two plants (Fig 3, plant No.1 and No.8) carring two copies of the SpI gene. No positive hybridization bands could be found in untransformed plants as a control. Thus, Southern blot analysis confirmed that the SpI gene had stably integrated into the genome of transgenic plants.

Inheritance of transgenes through sexual generation

After transplanting to soil, all transgenic rice plants grew well and produced normal seeds (Fig 1-F). Inheritance of the marker gene nptII was investigated. All the selfed seeds of T_1 generation, when sowed in solidified MS0K medium with 75 mg/L kanamycin, can produce some green shoots normally, whereas, all the control seeds from untransformed plants died. Segregation for kanamycin resistance were observed in 12 independently transformed plant lines (Tab 3). The results indicated that the segregating ratios in most of the lines tested fit the 3:1 model for single dominant gene inheritance. Exception was found in line X5, in which there were more sensitive plants than expected from the Mendelian model. The results of genetic analysis of the T_1 generation further demonstrated the stable incorporation of T-DNA into the rice genome and inheritance in the subsequent generations.

Insecticidal activity of T_1 transgenic plants

Our long-term goals are to improve the quality of rice by using an approach in which multiple disease- and insect-resistant traits can be introduced into the same lines [2]. In our experiment, the insecticidal activity of transgenic rice plants on LF and SSB wereinvestigated (Tab 4 and Tab 5). For LF insect-resistance test, the leaves of 117 transgenic plants from two transgenic lines of Xiushui 11 and Chunjiang 11 were infested with the larvae of the second instar stage of LF. Seven d after infestation, the insecticidal activity and the resistance level were examined (Tab 4). Average mortality of 38.4%-61.2% was observed in the two transgenic lines for the two rice varieties 7 d after treatment, whereas in the control plants, mortality was < 1% in most cases. Significant leaf damages

were observed in control plants as a result of LF feeding, whereas in transgenic plants, there was little detectable damage (Fig 1-C). For SSB insect-resistance test, stem cuttings and, in most cases, whole plants from eight transgenic lines (N_1 to N_8) carrying SpI were infested with the larvae of the second instar stage of SSB. The corrected mortality after 7 d of insect feeding was examined (Tab 5). Only larvae found inside the stems were recorded. Two to seven d after infestation, escape of insects from the stem was found in some transgenic plants infested and part of them died later (Fig 1-D). Corrected mortality of 36%-75% was observed in most of the transgenic lines (N_1, N_3-N_8), which were found to be highly toxic to SSB. These plants produce seeds normally after 30 d of treatment (Fig 1-F). One exception was found in transgenic plant line N_2. In contrast, all seed-derived plants as control growth abnormally after 30 d of treatment.

In conclusion, we have obtained over 970 transgenic rice plants by Agrobacterium-mediated transformation procedure. The transgenes in most of the plants analysed were inherited as Mendelian traits. Feeding assays with T1 plants from 10 independent transgenic lines confirmed that the transgenic plants were highly toxic to LF and SSB larvae.

ACKNOWLEDGMENTS

Tab 3. Genetic analysis of T1 generation of transgenic rice plants

		Number of T1 seedlings		Ratio of segregation	r value for	
Rice cultivar	Transfomants	Kanr	Kans	(Kanr /Kans)	c2	3:1 ratio
Xiushui 11	X1	21	9	2.3	0.400	0.55
	X2	23	7	3.	0.044	0.82
	X3	25	5	5.0	1.112	0.19
	X4	17	4	4.3	0.397	0.54
	X5	11	14	0.8	12.813	< 0.01
	X6	18	7	2.6	0.120	0.74
Chunjiang 11	C1	21	8	2.6	0.103	0.75
	C2	12	3	4.0	0.143	0.72
	C3	9	0	-	3.000	0.09
	C4	17	6	2.8	0.014	0.95
	C5	18	5	3.6	0.130	0.73
	C6	15	6	2.5	0.143	0.72

Tab 4. Insecticidal activity of T 1 transgenic rice on leaffolder larvae

Rice materials	Number of plants tested	Average mortality 7 d after treatment (%)	Resistance level
Control	40	0	Highly sensitive
Xiushui-*SpI*	78	61.2 ±10.7	Highly resistant
Chunjiang-*SpI*	39	38.4±7.6	Moderately resistant

Tab 5. Insecticidal activity of T1, transgenic rice (Xiushui 11) on striped stem borer larvae

Transgenic lines	Average weight of survived larvae in 7 d of treatment (mg)	Mortality within 7 d after treatment (%)	Corrected mortality (%)	Formation of flowers and seeds after 30 d of treatment
Control	81.2±10.9	2.7±0.3	0	Abnormal
N_1	34.7±4.8	65.4±10.6	64.4	Normal
N_2	64.5±7.0	18.4±2.8	16.1	Abnormal
N_3	72.9±9.4	48.7±11.2	47.3	Normal
N_4	51.8±12.9	67.6±10.4	66.7	Normal
N_5	23.7±8.9	75.2±14.1	74.5	Normal
N_6	48.3±7.2	45.8±4.9	44.3	Normal
N_7	52.4±8.7	37.6±10.8	35.9	Normal
N_8	38.6±11.5	70.5±18.	69.7	Normal

The authors are very grateful to Prof. TANG Zhen Hua and ZHUANG Pei Jun, Dr. WU Jun of Institute of Entomology, CAS for their useful suggestion and kind help in insect resistance tests. We also thank Prof. FANG Ping Yi for providing rice materials used in this experiment. Life Science Special Fund of CAS (No. Stz-3-03) Supported by the Ministry of Finance.

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