Breeding and Identification of Insect-Resistant Rice by Transferring Two Insecticidal Genes, sbk and sck

ZHANG Qi-jun1, #, LI Cong2, #, LIU Shao-kui2, LAI Dong2, Qi Qing-ming1, LU Chuan-gen1

(1Institute of Food Crops, Jiangsu Academy of Agricultural Sciences/Jiangsu High Quality Rice R&D Center, Nanjing 210014, China; 2College of Agriculture, Nanjing Agricultural University, Nanjing 210095, China; # These two authors contributed equally to this study)

Abstract: The plasmid of pCDMARUBA-Hyg, which contained two insect-resistance genes, sbk (modified from Cry1A(c)) and sck (modified from CpTI), was transformed into an Agrobacterium EHA105 for infection of the calli of a super japonica rice Nanjing 45. Primarily, using polymerase chain reaction (PCR) detection with the primers of sbk and sck genes, 42 positive transgenic plants that were marker-free and contained the two target genes were selected from 97 regenerated plants. Results of southern-blotting indicated that 23, 11, 5, 2 and 1 plants had one, two, three, four and five copies of the transformed genes, respectively. Analysis of reverse transcription PCR (RT-PCR) and Bt gene testing paper showed that 28 T3 generation plants derived from four transgenic plants having a single copy were insect-resistant. Feeding experiment with rice stem borer revealed that the insect resistance was greatly increased with the larva mortality ranging from 94% to 100%. In addition, among the transgenic plants, three T3 transgenic plants possessed some desirable characteristics for breeding and production, such as plant height, seed-setting rate, 1000-grain weight and larva mortality. The mechanism of insect resistance of Bt gene and its application in rice transgenic research were also briefly discussed.

Key words: Oryza sativa; sbk gene; sck gene; insect resistance; transgene; breeding

Rice is one of the most important food crops in the world, and also the staple food for more than one-third of the world’s population. However, rice is one of the grain crops most seriously attacked by pests. Estimated data showed that annual rice loss of the world due to pest accounts for more than 5% of the total output of rice (Wang et al, 2009). To control pests by conventional pesticides not only increases the cost of inputs, but also pollutes the ecological environment. By exogenous insect-resistance genes, breeding rice varieties with insect resistance can effectively reduce the loss and stabilize rice yield (Chen et al, 2009). To this end, many researchers have made unremitting exploration and innovation (Yang et al, 1989; Xie et al, 1991; Ghareyaze et al, 1997; Shu et al, 2000; High et al, 2004; Chen et al, 2005; Tang et al, 2006; Kim et al, 2009). However, most studies have focused on the genetic manipulation of a single gene, and a single insect-resistance gene is likely to lure insect resistance to the toxins (McGaughney and Whalon, 1992; Chen et al, 2011). Breeding lines with multiple insect-resistant mechanisms and genes that have different insect-resistant broad-spectrum and expressed simultaneously, can effectively reduce the generation of insect resistance (Cheng et al, 1998; Xiang et al, 1999; Feng et al, 2000; Bharathi et al, 2011; Yang et al, 2011).

Bt genes were preferred for insect-resistant transgenic study because their insect-resistant spectrum were almost covered the orders Lepidoptera, Coleoptera and Diptera (Yu, 2000). In addition, the cowpea trypsin inhibitor gene (CpTI) was another insect-resistance gene widely used in plant breeding because it could help the plant resist the larvae of Lepidoptera, Coleoptera and Orthoptera (Feng et al, 2000). Therefore, stacking both of insect-resistance genes in plants may greatly increase the insect resistance and gain everlasting resistance (Yu, 2000). Modified Cry1A(c) and CpTI were transferred into rice variety Minghui 86 and the transgenic rice plants showed strong resistance to target pest (Zhu, 2001). The result was further confirmed by many other studies (Li et al, 2002; Ma et al, 2003; Gao et al, 2006; Zhang et al, 2008; Zhang et al, 2011).
The annual planting area of japonica rice is approximately 1,800,000 hm² in Jiangsu Province, China, where suffers serious damage by insects such as *Cnaphalocrocis medinalis* and *Scirpophaga incertulas*. The existing transgenic rice plants in Jiangsu Province or its surrounding areas cannot effectively solve insect problem of japonica rice production in the middle and lower reaches of Yangtze River.

Nanjing 45, a japonica rice variety, was currently certificated and recommended as one of the main generalized cultivars in Jiangsu Province. In this study, we transferred two insect-resistance genes, *sbk* and *sck*, into Nanjing 45 to improve its resistance. This study would provide useful resources for rice breeding or even offer insect-resistant rice lines for rice breeding and production in future.

**MATERIALS AND METHODS**

**Plasmid vector and rice material**

The plasmid of pCDMARUBA-Hyg was provided by Prof. Zhu Zhen (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China), and it contained marker-free bivalent insect-resistance genes (Fig. 1). There are two insect-resistance genes (*sbk* and *sck*), and two selective markers on the vector, one is *hygromycin* gene used for selecting plant and the other is *kanamycin* gene used for screening bacterial strain. The acceptor material was a japonica rice cultivar, Nanjing 45.

**Genetically modified manipulation**

N6 was as basic medium, while induction and differentiation media were prepared according to the formula of Huang et al (2008), and general operation followed the method of Zhang et al (2008). Brown rice of Nanjing 45 were sterilized firstly with 70% ethanol for 5 min and then with 0.1% mercuric chloride for 20 min, finally washed with sterile distilled water for 5–8 times, each time for 2 min. Sterile seeds were inoculated on the induction medium, dark for 7–10 d to induce calli, and the calli were sub-cultured 1–2 times. Calli grown on a new medium for 2–3 d were dipped with the liquid of *Agrobacterium* for 20 min, then transferred to a common medium for 2 d, washed away the excess bacteria cultured; transferred to a recovery medium, dark for 20 d; and then transferred to a differentiation medium, replacing new medium every 10 d. Seedlings were transferred to a rooting medium when the length reached 1–2 cm, and then transplanted to pots with conventional water and fertilizer management when the seedlings were 7–10 cm long after adaptation for 2–3 d.

**Molecular detection of target gene**

**Polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) analysis**

The plasmid pCDMARUBA-Hyg mainly contains two insect-resistance genes (*sbk* and *sck*), also with a *hygromycin* selectable marker gene (Fig. 1). Procedure of PCR amplification was as follows: 94 °C for 5 min; 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, 35 cycles; finally 72 °C for 10 min. Two target genes and a selection marker primer sequences are shown in Table 1. PCR products were electrophoresed on 1% agarose gel and photographed.

Total RNA was extracted from rice leaves at the tillering stage. Reverse transcription and synthesis of cDNA first strand followed the instructions of the reverse transcription and synthesis of cDNA first strand kit (Nanjing Shengxing Biotechnology Co., Ltd, China) produced by Sigma Co., Ltd.

**Southern blotting**

The recovered PCR products with *sck* gene primer in plasmid were used as probe labeling. Total genomic DNA of positive plants hybridized after being digested with *BamH I* and electrophoretic transferred. It was operated according to the reference of DIG High Prime DNA Labeling and Detection Starter Kit II (Cat. No. 11585614810, Roche Co., Ltd).

**Detection of Bt gene**

The detection method of the colloidal gold test paper (Chongqing Jinbiao Biological Technology Co., Ltd,

---

![Fig. 1. Frame diagram of pCDMARUBA-Hyg plasmid (Ma et al, 2003, slightly modified).](image)

Mar, Nuclear matrix combination area sequence; T-nos, Rouge alkali synthetase terminator; *sck*, Modified from *CpTI*; Hyg, Hygromycin phosphotransferase gene; P-Iubt, Maize ubiquitin promoter; P-Actin, Rice actin promoter; *Kan*, Kanamycin gene; *Cry1Ac*, *Bacillus thuringiensis* gene 1A(c); CaMV35S, Cauliflower mosaic virus terminator; LB, T-DNA left border; RB, T-DNA right border.
China) was conducted according to its manuals. A test line and a control line on the test strip was positive (containing \textit{Bt} gene protein) and denoted as ‘+’. Only one detective line was negative (no \textit{Bt} poisonous protein) and denoted as ‘-’.

**Rice stem borer artificial feeding and agronomic traits investigation**

In this experiment, identification of artificial feeding was after the method of Gao \textit{et al} (2009). In order to collect \textit{Chilo supperssalis} eggs from natural disease fields, no pesticides were sprayed during the whole rice growth stage. Five plants were randomly selected and their 6-cm long bottom stalks were cut for experiment. After the eggs massively hatched, 10 rice stem borer larvae were inoculated in each stalk with a fine brush, then put into a small glass tube (15 mm × 150 mm) with 2 mL water. And the tube was sealed with nozzle filter paper and placed in the condition of 22−25 °C, relative humidity (RH) 90% for 5 d. Insect numbers of the death and the survival after 5 d of inoculation were recorded, and then the mortality (larva death rate, LDR) was calculated. Some agronomic characters, such as plant height, growth duration, number of filled grains per panicle, seed-setting rate and 1000-grain weight were measured. The japonica rice variety, Nanjing 45 (NJ45), as the control, was also surveyed.

**RESULTS**

**Obtaining of transgenic positive plants**

Ninety-seven regeneration plants of Nanjing 45 were obtained through callus screening and green seedling regeneration by \textit{Agrobacterium}-mediated technique. Results of PCR identification indicated that 42 regeneration individuals harboring the exogenous target genes, \textit{sbk} and \textit{sck}, was marker-free (Fig. 2). Southern blotting showed that the exogenous insect-resistance genes have been integrated into the genome of Nanjing 45. Of the 42 individuals, 23, 11, 5, 2 and 1 plants had one, two, three, four and five copies of the transformed genes, respectively (Fig. 3).

**Expression of insect-resistance genes**

Twenty-eight positive transgenic plants that had single copy were randomly selected from the third generation (T₃), and extracted RNA for RT-PCR. The characteristic strips of exogenous genes \textit{sbk} and \textit{sck} were evident

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>Size of fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{sck}</td>
<td>AAAATGAAGAGCACCATTTC</td>
<td>TCTAGAGTTCACTTCTTCATC</td>
<td>415</td>
</tr>
<tr>
<td>\textit{sbk}</td>
<td>TGCAAGAAGCATTCAAGAGTG</td>
<td>ACACCTGACCTAGTGGGC</td>
<td>743</td>
</tr>
<tr>
<td>\textit{Hyg}</td>
<td>TACACAGCCATCGGTCCAGA</td>
<td>TAGGAGGCGGGGTGGATGTC</td>
<td>832</td>
</tr>
</tbody>
</table>

**Table 1. Primer sequence of genes or linkage markers.**

Fig. 2. PCR identification of T₃ transgenic plants.

M, 100 bp DNA ladder marker; A, B and C, Amplification plasmid DNA with the primers of \textit{sck}, \textit{sbk} and \textit{Hyg}, respectively. Lanes 1 to 42, Positive transgenic plants.

Fig. 3. Southern blotting of partial T₀ transgenic plants with \textit{sck} gene full-length DNA as hybridization probe.

CK, Nanjing 45; Lanes 1 to 42, T₀ transgenic plants which have one to five copies of exogenous \textit{sck} gene.
(Fig. 4), which showed that the exogenous genes not only integrated into the 28 plants, but also expressed effectively and stably. Analysis with \textit{Bt} gene testing paper also indicated that the two insect-resistance genes were efficiently and stably expressed (Fig. 5). All of these results ensured the insect resistance of transgenic plants and their role in future application.

**Insect-resistance and agronomic traits of transgenic plants**

Agronomic characteristics of transgenic plants and the control, as well as larva mortality of the stem borer, were summarized in Table 2. Results indicated that the larva mortalities of the rice stem borer of T3 transgenic lines ranged from 94% to 100%, showing that the insect resistance of transgenic plants was greatly increased. Individuals T45-2, T45-11 and T45-14 showed the similar agronomic traits with Nanjing 45, including growth duration, number of filled grains per panicle, seed-setting rate and 1000-grain weight. This means that those transgenic lines kept excellent characteristics of Nanjing 45. The insect resistance of other transgenic lines was also significantly increased, however, their agronomic characteristics showed some differences compared to those of Nanjing 45.

**DISCUSSION**

After being preyed by rice stem borer, the toxin protein crystal from \textit{Bacillus thuringiensis}, is dissolved and activated by the effect of intestinal alkaline protease. It releases the core peptide toxins, which act on the intestinal epithelial cells and cause

Table 2. Agronomic characters and larva mortality of partial T3 transgenic lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Origination (T0)</th>
<th>Plant height (cm)</th>
<th>Growth duration (d)</th>
<th>Grain number per panicle</th>
<th>Seed-setting rate (%)</th>
<th>1000-grain weight (g)</th>
<th>Larva mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NJ45 (CK)</td>
<td></td>
<td>105.1 ± 0.3 a</td>
<td>154</td>
<td>119.2 ± 0.1 b</td>
<td>91.6 ± 0.1 b</td>
<td>28.4 ± 0.1 c</td>
<td>6.0 ± 0.9 a</td>
</tr>
<tr>
<td>T45-1</td>
<td>T0-9</td>
<td>107.2 ± 1.2 c</td>
<td>150</td>
<td>123.6 ± 5.0 ab</td>
<td>90.4 ± 0.3 c</td>
<td>27.8 ± 0.2 c</td>
<td>94.0 ± 0.6 b</td>
</tr>
<tr>
<td>T45-2</td>
<td>T0-9</td>
<td>104.5 ± 0.3 c</td>
<td>154</td>
<td>120.1 ± 0.8 ab</td>
<td>92.0 ± 0.1 b</td>
<td>28.4 ± 0.2 c</td>
<td>100.0 ± 0.0 b</td>
</tr>
<tr>
<td>T45-8</td>
<td>T0-15</td>
<td>108.4 ± 1.2 b</td>
<td>149</td>
<td>113.5 ± 5.3 c</td>
<td>89.8 ± 0.5 c</td>
<td>28.9 ± 0.3 b</td>
<td>98.0 ± 0.5 b</td>
</tr>
<tr>
<td>T45-11</td>
<td>T0-21</td>
<td>105.2 ± 0.3 d</td>
<td>153</td>
<td>122.3 ± 1.3 ab</td>
<td>91.8 ± 0.2 b</td>
<td>28.5 ± 0.1 c</td>
<td>100.0 ± 0.0 b</td>
</tr>
<tr>
<td>T45-14</td>
<td>T0-21</td>
<td>103.7 ± 0.4 e</td>
<td>155</td>
<td>119.8 ± 1.1 ab</td>
<td>92.2 ± 0.2 b</td>
<td>28.3 ± 0.1 cd</td>
<td>100.0 ± 0.0 b</td>
</tr>
<tr>
<td>T45-19</td>
<td>T0-40</td>
<td>97.2 ± 0.2 f</td>
<td>148</td>
<td>107.7 ± 4.7 d</td>
<td>93.1 ± 0.4 a</td>
<td>28.1 ± 0.2 d</td>
<td>96.0 ± 0.6 b</td>
</tr>
<tr>
<td>T45-27</td>
<td>T0-40</td>
<td>109.8 ± 1.3 a</td>
<td>158</td>
<td>125.3 ± 7.3 a</td>
<td>87.6 ± 0.5 d</td>
<td>29.3 ± 0.3 a</td>
<td>98.0 ± 0.5 b</td>
</tr>
</tbody>
</table>

The data presented are the mean ± SE. The same lowercase letters after numbers within a column indicate no significant differences between materials \((P > 0.05)\).
cell swelling and lysing. Furthermore, it causes intestinal paralysis and perforation of the insect, and the ions and osmotic balance of gastrointestinal cells is destroyed so that the insect is killed. BT proteins can be dissolved completely in a few seconds under the effect of acidic gastric juice after it goes into the intestines and stomach of mammals. For this reason, it is harmless to human, birds, fish and non-target insects, and it can be dissolved easily and has little environment impact on the soil (Zhang, 2010). According to the study, the expression level of insecticidal protein gene of pest-resistant transgenic rice was higher at the earlier growth stage than at the later growth stage, and the expression in leaves was greater than that in stems, while the expression level was barely detected in grains (Li et al, 2004). Therefore, so far, Bt gene is probably the most widely used and the safest pest-resistance foreign gene in the genetic engineering of crops (Wunn, 1996; Ghareyaze et al, 1997; Xiang et al, 1999; Shu et al, 2000).

Although there is no commercialized transgenic rice in China, a few reports are available about Bt pest-resistant transgenic rice (Yang et al, 1989; Xie et al, 1991; Xiang et al, 1999). Those studies mainly used one or two Bt genes to resist rice stem borer, and they found the insect resistance of the transgenic rice was improved (Li et al, 2007; Cai et al, 2008; Zhang et al, 2008). Some other researchers selected different pest-resistance gene combinations to improve the pest resistance of rice and also obtained remarkable achievements (Cheng et al, 1998; Maqbool and Christou, 1999; Tu et al, 2000; Lin et al, 2002; Li et al, 2008). In the present study, we selected the modified Bt gene and the cowpea trypsin inhibitor gene (CpTI) for genetic transformation and obtained 42 non-antibiotics transgenic rice plants, of which the pest resistance reached 94%–100%. It is suggested that lines T45-2, T45-11 and T45-14, possessing the similar excellent agronomic characteristics with high-yielding japonica cultivar, Nanjing 45, seem to be elite lines for rice breeding and production due to their desirable agronomic traits.

CONCLUSIONS

Methods including PCR, RT-PCR, southern blotting and Bt gene testing paper were applied to select the transgenic progenies without antibiotic genes in this experiment. A total of 42 positive plants (23, 11, 5, 2 and 1 plants had one, two, three, four and five copies of the transformed genes, respectively) were obtained. Results of artificial inoculating experiment showed that the insect resistance was greatly enhanced by introducing the two insect-resistance genes. In the present study, we have selected three elite lines, T45-2, T45-11 and T45-14, which offered germplasm for insect resistance breeding and provided elite varieties for japonica rice production.

ACKNOWLEDGEMENTS

This research was financially supported by the Natural Science Foundation of Jiangsu Province, China (Grant No. BK2008348) and the China National Science & Technology Major Project for Breeding New Plant Varieties of Genetically Modified Organisms (GMOs) (Grant No. 2008ZX08001-004).

REFERENCES


Cheng X Y, Sardana R, Kaplan H, Altosaar I. 1998. Agrobacterium-transformed rice plants expressing synthetic cry1A(b) and cry1A(c) genes are highly toxic to striped stem borer and yellow stem borer. Proc Natl Acad Sci USA, 95(6): 2767–2772.


Wunn J. 1996. Transgenic indica rice breeding line IR58 expressing a synthetic Cry1A(b) gene from *Bacillus thuringiensis*. *Nat Biotechnol*, 14: 171–176.


