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

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Abstract: The plasmid of pCDMARUBA-Hyg, which contained two insect-resistant genes, \textit{sbk} [modified from \textit{Cry1A(c)}] and \textit{sck} (modified from \textit{CpTI}), was transformed into an agrobacterium EHA105 for infection of the calli of a super japonica rice Nanjing 45. Plants regenerated from the transformation were subjected to all kinds of gene verifications and selection. Using PCR detection with the primers of \textit{sbk} and \textit{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 RT-PCR and \textit{Bt} gene testing paper showed that 28 \textit{T}_3 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 nymph mortality ranging from 94% to 100%. In addition, among the transgenic plants, three \textit{T}_3 transgenic plants had many desirable characteristics for production. The mechanism of insect-resistance of \textit{Bt} gene and its application in rice transgenic research were also briefly discussed.

Key words: \textit{Oryza sativa} L; \textit{sbk} gene; \textit{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. According to incomplete data, the world’s annual rice losses due to pests, accounts for more than 5% of the total output of rice (Wang et al, 2009). To control pests by conventional pesticides not only increase the cost of inputs, but also easy to pollute the ecological environment. Rely on genetic transformation of exogenous insect-resistant genes, breeding rice varieties with insect-resistant ability can effectively reduce losses and stabilize rice yield (Chen et al, 2009). For this reason, 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, these studies have focused on the genetic manipulation of a single gene, and a single insect-resistant gene likely to lure insects resistant to the toxins (McGaughey and Whalon; 1992; Chen et al, 2011). Breeding lines with multiple different insect-resistant mechanism and insect spectrum genes and expressed in genetically modified crops, can effectively relieve 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).

\textit{Bt} genes were regarded as preferred genes for insect-resistant transgenic study because their insect-resistant spectrum were almost covered the orders \textit{Lepidoptera}, \textit{Coleoptera} and \textit{Diptera} (Yu, 2000). In addition, the cowpea trypsin inhibitor gene (\textit{CpTI}) was another insect-resistant gene widely used in plant breeding because it could kill the larvae not only \textit{Lepidoptera} and \textit{Coleoptera}, but also \textit{Orthoptera} (Feng et al, 2000). Therefore, polymerizing these two kinds of insect-resistant genes in plant may greatly increase the insect-resistant ability and gain everlasting resistance (Yu, 2000). Modified \textit{CryIA(c)} and \textit{CpTI} were transferred in Minghui 86 and the transgenic rice plants showed strong resistance to target pest (Zhu, 2001). Thereafter, this conclusion was 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 planting area of japonica rice in Jiangsu Province is approximately 1 800 000 hm\textsuperscript{2} per year, where the damage by insects such as \textit{Cnaphalocrocis medinalis} and \textit{Scirpophaga incertulas} is very serious. The existing transgenic rice plants seemed to be unsuitable for rice production in Jiangsu Province or its surrounding areas, and cannot effectively solve this
problem of japonica rice production in the middle and lower reaches of Yangtze River.

Nanjing 45, a japonica rice cultivar, was newly certificated by the governor and recommended as one of the main generalized cultivars in Jiangsu Province. In this study we transferred two insect-resistant genes, sbk and sck, into Nanjing 45 to improve its resistance. From this study, it would provide useful resources for rice breeding or even offer insect-resistant rice lines for rice 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-resistant genes (Fig. 1). There are two insect-resistant 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 plasmid vector can be used for genetic modification of plant with marker-free insect-resistant because its structure is double-border design. The genetically modified acceptor material in this study is a japonica rice cultivar, Nanjing 45.

Genetically modified manipulation

N6 was as basic medium, while induction and differentiation medium were utilized according the formula of Huang’s (Huang et al, 2008), and general operation was in accordance with the process of Zhang’s (Zhang et al, 2008). Mature seeds of Nanjing 45 after being ground and shelled were firstly with 70% ethanol disinfection for 5 min and then with 0.1% mercuric chloride for 20 min, finally washed with sterile distilled water for 5 to 8 times, each time for 2 min. Sterile seeds were inoculated on induction medium, dark for 7−10 d to induce callus, which was sub-cultured 1−2 times. Callus grown 2−3 d on a new medium was 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 recovery medium, dark for 20 d; and then transferred to differentiation medium, replacing a new medium per 10 d. Seedlings was transferred to rooting medium when the length reached to 1−2 cm, and then transplanted to pots with conventional water and fertilizer management when the seedlings was 7−10 cm long after adaptation for 2−3 d.

Molecular detection of target gene

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

The marker-free plasmid of pCDMARUBA-Hyg mainly contains two insect-resistant 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 samples were electrophoresed by 1% agarose gel and photographed.

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

Southern blotting

Products recovered from the PCR amplification with sck gene primer in plasmid were used as probe labeling. Total genomic DNA of positive plant will be 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, Chongqing, China) was conducted according to its manuals. A test line and a control line on the test strip was positive (containing Bt gene protein), denoted it as “+”. Only one detective line was negative (no Bt poisonous protein), denoted it as “−”.

Fig. 1. Frame diagram of pCDMARUBA-Hyg plasmid (Ma et al, 2003).

sbk and sck, modified from Cry1Ac and CpTI, respectively; Hyg, Hygromycin Phosphotransferase.
Table 1. Primer sequence of genes or linkage markers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size of fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sck</td>
<td>AAAATGAAGAGCACCATCTTC</td>
<td>TCTAGAGTTTCATTTTCATC</td>
<td>415</td>
</tr>
<tr>
<td>Shk</td>
<td>TGCAGAGAGCTTCAGAGTAG</td>
<td>ACACCTGACCTAGTTGAGC</td>
<td>743</td>
</tr>
<tr>
<td>Hyg</td>
<td>TACACAGCCATCGGTCCAGA</td>
<td>TAGGAGGCGTGGATATGTC</td>
<td>832</td>
</tr>
</tbody>
</table>

Rice stems borer artificial feeding and agronomic traits investigation

In this experiment, identification of artificial feeding was after the method of Gao’s (Gao et al, 2009). In order to collect Chilo suppressalis eggs from natural disease field, no pesticides was sprayed during the whole rice growing stage. Five plants were randomly selected and their 6 cm-long bottom stalks were cut for experiment. After the egg 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, sealed with nozzle filter paper and placed in the condition of 22–25 °C, relative humidity (RH) 90% for 5 d. Insect number 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 period, number of filled grains per panicle, setting percentage, 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

Ninty-seven regeneration plants of Nanjing 45 were obtained through callus screening and green seedling regeneration by agrobacterium-mediated technique. Results of PCR identification indicated that 42 regeneration individuals harboring the exogenous target genes, \( sbk \) and \( sck \), being marker-free (Fig. 2). Southern blotting showed that the exogenous insect-resistant genes had 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-resistant genes

Twenty-eight positive transgenic plants that had single copy were randomly selected from the third generation (\( T_3 \)), and extracted RNA for reverse transcription-polymerase chain reaction (RT-PCR). The characteristic strips of exogenous genes \( sbk \) and \( sck \) were evident (Fig. 4), which showed that the exogenous genes not only integrated into the 28 plants, but also expressed effectively and stably. Analysis with \( Bt \) gene testing paper also indicated that the two insect-resistant genes were normally and stably expressed (Fig. 5). All of these results ensured the insect-resistant abilities of transgenic plants and their
Insect-resistant ability and agronomic traits of transgenic plants

Comparisons of agronomic characteristics of transgenic plants and the control in the feeding rice stem borer were summarized in Table 2. Results indicated that the insecticidal effects were 97% to 100% on the rice stem borer, so the insect-resistant ability of transgenic plants was greatly increased. Individuals T45-2, T45-11 and T45-14 showed similar traits with Nanjing 45, including plant height, growth period, 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. Insect-resistant ability of other transgenic lines was also 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 *Bacillus thuringiensis*, is dissolved and activated in the effect of intestinal alkaline protease. It releases the core peptide toxins, which act on the intestinal epithelial cells and causes cell swelling and lysing. Furthermore, it causes intestinal paralysis and perforation of the insect, and the ions and osmotic balance of gastrointestinal cells is destructed 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 intestines and stomach of mammals. For this reason, it is harmless to mammals, birds, fishes 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

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Origination (T3)</th>
<th>Plant height (cm)</th>
<th>Growth period (d)</th>
<th>Grain number per panicle</th>
<th>Seed-setting rate (%)</th>
<th>1000-grain weight (g)</th>
<th>Larvae mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NJ45 (CK)</td>
<td>105 ± 0.13 d</td>
<td>154</td>
<td>119 ± 0.10 ab</td>
<td>91.6 ± 0.07 c</td>
<td>28.4 ± 0.07 c</td>
<td>6 ± 0.89 a</td>
<td></td>
</tr>
<tr>
<td>T45-1</td>
<td>T9-9</td>
<td>107 ± 1.20 c</td>
<td>150</td>
<td>123 ± 5.03 ab</td>
<td>90.4 ± 0.29 d</td>
<td>27.8 ± 0.21 e</td>
<td>94 ± 0.55 b</td>
</tr>
<tr>
<td>T45-2</td>
<td>T9-9</td>
<td>104 ± 0.26 d</td>
<td>154</td>
<td>120 ± 8.44 ab</td>
<td>92.0 ± 0.12 bc</td>
<td>28.4 ± 0.16 c</td>
<td>100 ± 0.00 b</td>
</tr>
<tr>
<td>T45-8</td>
<td>T9-15</td>
<td>108 ± 1.18 b</td>
<td>149</td>
<td>113 ± 5.31 c</td>
<td>89.8 ± 0.51 e</td>
<td>28.9 ± 0.34 b</td>
<td>98 ± 0.45 b</td>
</tr>
<tr>
<td>T45-11</td>
<td>T9-21</td>
<td>105 ± 0.32 d</td>
<td>153</td>
<td>122 ± 1.30 ab</td>
<td>91.8 ± 0.18 bc</td>
<td>28.5 ± 0.07 c</td>
<td>100 ± 0.00 b</td>
</tr>
<tr>
<td>T45-14</td>
<td>T9-21</td>
<td>103 ± 0.37 e</td>
<td>155</td>
<td>119 ± 1.14 b</td>
<td>92.2 ± 0.21 b</td>
<td>28.3 ± 0.08 cd</td>
<td>100 ± 0.00 b</td>
</tr>
<tr>
<td>T45-19</td>
<td>T9-40</td>
<td>97 ± 0.19 f</td>
<td>148</td>
<td>107 ± 4.74 d</td>
<td>93.1 ± 0.44 a</td>
<td>28.1 ± 0.19 d</td>
<td>96 ± 0.55 b</td>
</tr>
<tr>
<td>T45-27</td>
<td>T9-40</td>
<td>109 ± 1.34 a</td>
<td>158</td>
<td>125 ± 7.26 a</td>
<td>87.6 ± 0.47 f</td>
<td>29.3 ± 0.31 a</td>
<td>98 ± 0.45 b</td>
</tr>
</tbody>
</table>
insecticidal protein gene of pest-resistant transgenic rice was higher in earlier growing stage than in later growing stage, and the expression in leaves was greater than that in stems, while the expression product was not detected in grains (Li et al, 2004). Therefore, Bt gene is probably the most widely used and the safest pest-resistant 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 transgenesis pest-resistant 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 insect-resistant ability of the transgenic rice was improved (Li et al, 2007; Cai et al, 2008; Zhang et al, 2008). Some other researchers selected different pest-resistant gene combinations to improve the pest-resistant ability of rice (Cheng et al, 1998; Maqbool and Christou, 1999; Tu et al, 2000; Lin et al, 2002; Li et al, 2008). Similarly, they obtained remarkable effects. The present study selected the modified Bt gene and the cowpea trypsin inhibitor gene (CpTI) for genetic transformation. This study obtained 42 non-antibiotic transgenic rice plants, of which the ability of pest-resistant reached 97–100%. It is suggested that lines T45-2, T45-11 and T45-14, possessing the same excellent agronomic characteristics with high-yield japonica cultivar, Nanjing 45, seem to be elite lines for rice breeding and production since their suitable agronomic traits.

CONCLUSION

Methods including PCR, RT-PCR, southern blotting and Bt gene testing paper were used to screen the transgenic progenies without antibiotic gene 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. Result of artificial inoculating experiment proved that the insect-resistant ability was greatly enhanced by introducing the two insect-resistant genes. The present research selected three elite lines, T45-2, T45-11 and T45-14, which offered resources for insect-resistant breeding and provided elite rice varieties for japonica rice production.

ACKNOWLEDGMENTS

This research was financially supported by the Natural Science Foundation of Jiangsu Province (BK2008348) and China National Science & Technology Major Project for Breeding New Plant Varieties of Genetically modified organisms (GMOs) (2008ZX08001-004).

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