Rice Science, 2009, 16(3): 181–186

DOI: 10.1016/S1672-6308(08)60077-3

Development of Marker-Free Transgenic *Cry1Ab* Rice with Lepidopteran Pest Resistance by *Agrobacterium* Mixture-Mediated Co-transformation

QI Yong-bin[1, 2], YE Sheng-hai[1], LU Yan-ting[1], JIN Qing-sheng[1], ZHANG Xiao-ming[1]
([1]*Institute of Crop and Nuclear Technology Utilization, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China;* [2]*Biotechnology Institute, Zhejiang University, Hangzhou 310029, China*)

Abstract: *Cry1Ab* gene was transformed into four rice varieties, Zhejing 22, Zhejing 27, Jiahua 1 and Xiushui 63 mediated by *Agrobacterium*-mixture co-transformation. Rice genotype had an important effect on callus induction and transformation efficiency. Different mixtures of *Agrobacterium* strains (EHA105 and EHA101) contained *Hpt* and *Cry1Ab* genes resulted in different frequencies of resistant calli. There was no correlation between the frequency of transformants with the ratio of the *Agrobacterium* strain mixture contained *Hpt* and *Cry1Ab* genes. A total of 509 transgenic plants were obtained from the four rice varieties, and 272 T_2 progenies were analyzed for *Cry1Ab* and *Hpt* genes. PCR analysis revealed that 412 regenerated plants were *Hpt* positive (80.94%), 62 plants were also *Cry1Ab* co-transformants (15.05% in total frequency), and 42 plants among the 272 T_2 progenies were *Cry1Ab* positive but *Hpt* negative. This suggests that marker-free transgenic plants could be produced by co-transformation mediated by mixed *Agrobacterium* strains with the selectable marker gene and target gene. Southern blot analysis of five independent marker-free T_2 transgenic lines co-transformed from Zhejing 22 showed that *Cry1Ab* gene had been inserted into rice genome with a single copy. The transgenic plants showed significantly stronger resistance to lepidopteron than the non-transgenic plants under no application of insecticides against lepidopteron.
Key words: rice (*Oryza sativa*); *Agrobacterium* mixture-mediated co-transformation; *Cry1Ab* gene; *Hpt* gene; gene transfer; marker-free transgenic plants; pest resistance;

Rice is one of the most important food crops in China with annually acreage of 30 million hectares, accounting for about one third of total cereal area, and producing about 45% of total cereal production. However, rice production suffered from insect damage, causing obvious yield loss. It is estimated that the acreage of rice damaged by insects and diseases surpassed 8.5 million hectares during 2001–2004 in China. Rice planthopper, stem borer and leaf folder are the major insects in China, occupying 72.5% of the total acreage of rice infested by insects and diseases [1]. Due to the lack of genetic resources for insect resistance, insect-resistant rice is difficult to be obtained by traditional breeding methods. However, the transgenic approach to improve insect resistance has become practicable in rice breeding since the first transgenic rice was obtained by the *Agrobacterium*-mediated method [2].

Many insect-killing proteins are produced by *Bacillus thuringiensis* (BT), which appear in the crystalform. These BT proteins can be hydrolyzed by proteinase in the intestines of insects and change into smaller toxic multi-peptides after insects have fed them. The toxic multi-peptides might interact with special acceptor(s) on intestines epithelium cells in insects, cause some non-special eyelets in the cell membrane, disturb the infiltration balance of cell, lead to cell decomposition, consequently result in insect death. Transgenic rice with the *Cry1Ab* gene encoding BT protein could be lethal to Lepidopteran insects including *Tryporyza incertulas*, *Chilo suppressalis*, *Cnaphalocrocis medinalis* and *Sesamia inferens*. Therefore, *Cry1Ab* gene transformation is a powerful and practical tool to prevent rice from insect damage [3].

Many transgenic rice plants have been obtained by using constitutive expression promoters, such as CaMV35S and Actin 1, which promote target gene expression in most (if not all) tissues of transgenic plants during growth [4-5]. Nevertheless, the herbicide-resistant and antibiotic marker genes are normally used as selectable markers in rice transformation, and selectable marker genes are inserted into plant genome

Received: 16 June 2008; **Accepted:** 12 April 2009
Corresponding author: ZHANG Xiao-ming (xmzhang@mail.hz.zj.cn)

as native DNA. This leads to biosafety concerns about the use of these genes in transgenic plants. Thus, some transformation techniques such as Cre/Lox [6], FLP-FRT [7], R/RS [8], Ac/Ds [9] and co-transformation [10-11] are developed to eliminate selectable marker genes and to obtain safe transgenic rice. In this study, one recombination plasmid, which harbors *Cry1Ab* gene without selectable marker gene was placed under the control of the Rubisco promoter to ensure that the BT protein only expresses in the green tissues of transgenic plants, the other recombination plasmid that only contains the *Hpt* gene, was co-transformed into rice calli. In the transgenic plants co-transformed by the *Hpt* and *Cry1Ab* genes, two genes were randomly inserted into different loci in T_0 rice genome, so the *Hpt* gene and *Cry1Ab* gene would be separated in the transgenic progenies. The *Cry1Ab* gene from transgenic rice plants was driven by Rubisco promoter, which can cause the expression of *Cry1Ab* gene only in green tissues or organs such as stem and leaf, but not in seed and pollen of transgenic plants [12].

MATERIALS AND METHODS

Rice materials and plasmids

Four japonica rice varieties, Zhejing 22, Zhejing 27, Xiushui 63 and Jiahua 1, widely cultivated in Zhejiang Province, China were used. The plasmid of pPZP201-RubS-*Cry1Ab* was constructed in the Professor Muthukrishnan's Laboratory, Biochemistry Department, Kansas State University, USA (Fig. 1). The *Cry1Ab* gene is driven by a Rubisco promoter (cloned from the plasmid pSBG700 [13]) and terminated by a nopaline synthase (nos) terminator, and no selectable marker gene was constructed on the vector.

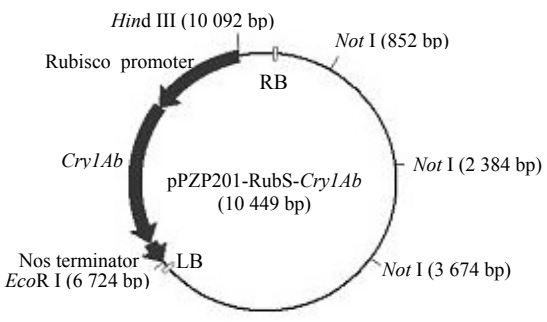

Fig. 1. Construction of the plasmid pPZP201-RubS-*Cry1Ab*.

Agrobacterium strain culture and co-transformation

Plasmids pCAMBIA1300 and pPZP201-RubS-*Cry1Ab* were introduced into *Agrobacterium* strains EHA105 and EHA101, respectively, by electroporation to get strains pCAMBIA1300-EHA105 and pPZP201-RubS-*Cry1Ab*-EHA101. These two strains were then incubated on a liquid LB medium supplemented with 50 mg/L kanamycin and 50 mg/L streptinomycin. The strain concentrations were adjusted to an OD_{600} in the range of 0.1 to 1.0 using *Agrobacterium* suspension media, and then mixed at different ratios. The mixtures were used to co-transform rice calli. The callus induction and co-transformation procedure were as described by Komari et al [10]. Mature embryo derived rice calli were co-cultured with the mixtures at different ratios for 2–3 days, and then transferred to a selection medium containing 50 mg/L hygromycin. After 6–8 weeks, the resistant calli produced on the selection medium were transferred to a redifferentiation medium to regenerate transformed plantlets.

PCR analysis and Southern blot

Rice genomic DNA was extracted from rice leaves using the CTAB method. PCR analysis was performed using the primers of 5′-atcggcgagtacttcta cacagcc-3′ and 5′-ctcgtgctttcagcttcgatgtag-3′ to detect *Hpt* gene, and the primers of 5′-caccacagaacaacaat gtgccacc-3′ and 5′-ctgtctagagtactcagcctcgaaggtaa-3′ to detect *Cry1Ab* gene. A 20 μL mixture of 20 ng of template DNA, 2.0 μL of 10×PCR buffer, 0.6 μL of 2 mmol/L dNTPs, 1.2 μL of 25 mmol/L $MgCl_2$, 0.4 μL of each of 10 μmol/L primers and 1 U of *Taq* DNA polymerase was prepared for PCR analysis. The PCR procedures for the two genes were the same: one cycle at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified fragments are 881 bp for *Hpt* gene and 604 bp for *Cry1Ab* gene, respectively. Southern blot analysis was used to determine the copy number of *Cry1Ab* gene in transgenic plants. Ten-microgram genomic DNA per sample was digested with *Eco*R I and separated on a 1.0% agarose gel, and then transferred to a nylon membrane. The probe was obtained from a PCR-amplified fragment of *Cry1Ab* gene and random-