Korean J. Plant Res. 20(3) : 242~246 (2007)

Agrobacterium-mediated Transformation of Rice 'Ilmibyeo' using *HPT* Selection Marker Gene

Jia Guo, Joon-Hyeong Cho[1], Hye Jeong Jo, Eun Soo Seong and Myeong-Hyeon Wang*
School of Biotechnology, Kangwon National University, Chuncheon, 200-701, Korea
[1]Department of Plant Biotechnology, Dongguk University, Seoul 100-715, Korea

Abstract - This study was conducted to produce the transgenic plant of rice. We obtained *Agrobacterium* AGL1 harbaring pCambia1300 vector with *HPT* gene. We carried out PCR analysis of 22 ea putative transgenic rice to investigate transformed lines. The 3 ea transgenic lines were detected insertion of HPT gene. Transgenic lines selected from PCR analysis were performed by Southern blot. From Southern blot, we obtained that two transgenic lines detected single band. We are going to study the method improving of cotransformation as well as transformation efficiency in rice.

Key words - *Agrobacterium* EHA 105, HPT gene, PCR analysis, Southern blot

Introduction

Rice is the important crop for more than half of the world population and has also become a model monocot system for genetic and functional genomic studies. Considerable efforts have been preceded toward the improvement of agronomic traits of rice by biotechnological techniques for recent years. Asian cultivated rice (*Oryza sativa* L.) composed of two major groups, which are known by the subspecies names *indica* (*Oryza sativa* ssp. *indica*) and *japonica* (*Oryza sativa* ssp. *japonica*). The indica subspecies is the most widely cultivated form of rice produced worldwide. The subspecies-specific genes have been identified in these subspecies and the sequence divergence revealed by whole genome sequencing of the two subspecies of cultivated rice (Yu *et al.*, 2005; *Zhang et al.*, 2005).

Productions of transgenic plants are important for plant science including plant molecular breeding, biosynthesis of industrial and pharmaceutical products and functional genomics. In contrast to direct gene transfer systems containing of DNA uptake via polyethylene glycol (PEG), electroporation and particle bombardment, *Agrobacterium*-mediated transformation has many advantages, such as low copy number of the transgene, and stable inheritance of transgenes in a Mendelian fashion (Gelvin, 2003). Many transformation systems have been reported for a wide range of plant species because of development for binary vector system (Hoekema *et al.*, 1983) and leaf disc transformation (Horsh *et al.*, 1985). Although the natural hosts of *Agrobacterium tumefaciens* are limited to certain dicotyledonous plants and some gymnosperms (De Cleen and De Ley, 1976), *Agrobacterium*-mediated transfor-mation has successfully been established for many monocotyl-edonous plants, including rice (Hiei *et al.*, 1994), maize (Ishda *et al.*, 1996), barley (Tingay, 1997) and wheat (Cheng *et al.*, 1997). Recently, non-tissue culture based 'in planta' transformation systems have been developed for *Arabidopsis* and a few other species (Bent, 2000). However, most *Agrobacterium*-mediated transformations are carried out using *in vitro* tissue culture. Thus, transformation efficiency highly depends on the regeneration abilities of genotypes and explants. However, transformation in *indica* rice remains difficult. There have been a few reports of successful transformation of *indica* rice by *Agrobacterium* (Khanna and Raina, 2002; Mohanty *et al.*, 2002). These transformation conditions either showed low transformation efficiency or were applicable to only limited *indica* varieties. Recently, a highly efficient system for *Agrobacterium*-mediated transformation of *indica* rice has been established (Lin and Zhang, 2005). The establishment of a highly efficient *Agrobacterium*-mediated transformation system in the *japonica* subspecies of rice (Hiei *et al.*, 1994) has accelerated. Remarkable attention is being paid to the functions of rice genes controlling various traits following the project of rice genomic sequences and to establish a stable transformation system.

*Corresponding author. E-mail : mhwang@kangwon.ac.kr

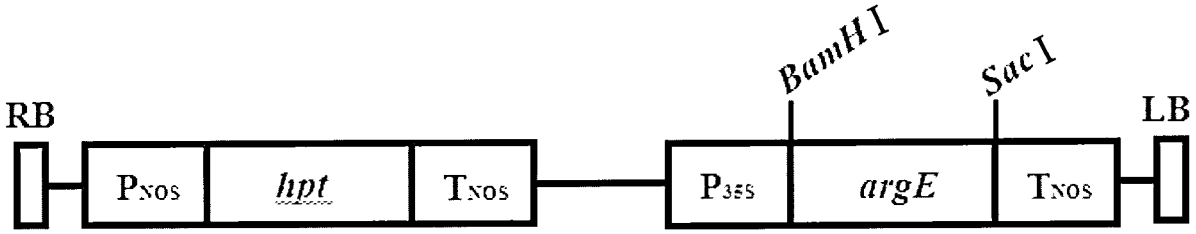

Fig. 1. Schematic representation of the T-DNA region of pCambia1300 plasmid.

Materials and Methods

Agrobacterium-mediated transformation

Single bacterial colony of *Agrobacterium* strain AGL1 was incubated in AB medium (AB buffer and salt, 5g/l glucose, 15g/l Bacto-agar, 50mg/l spectinomycin, 10mg/l tetracyclin, pH 7.2) at 28℃ for 72h. Bacterial cells were collected by scraping and resuspending in AAM medium (AA macro and micro salt, Amino acid stock, MS vitamins, 500mg/l casamino acid, 68.5g/l sucrose, 36g/l glucose) containing 100mM acetosyringone and the final concentration of *Agrobacterium* was adjusted to 5×10^9 cells/ml. The *Agrobacterium* strain AGL1 was mixed at 1 : 1 ratio before being used for transformation.

Embryogenic calli derived from the scutellum of mature rice seeds, "Ilmibyeo" was used for transformation according to the method (Hiei *et al.*, 1994) with slight modification. Embryogenic calli selected were mixed with Agrobacteria in 25ml AAM solution containing around 5×10^9 cell/ ml and incubated for 20 min at room temperature. The calli were then plated on NB3 medium (N6 4g, casein 0.3g, proline 2.9g, sucrose 30g, 2.4-D 2ml, glutamine 0.5g, N-B micro 1ml, N-B vts 1ml, myo-inositol 0.1g, pH 5.8, gelrite 2.5 g) for co-cultivation at 25℃ in dark condition for 3 days. After washing to remove the overgrown Agrobacteria using cefotaxime solution (250mg/l in dd H_2O), the calli were transferred to 2N6-CH medium (2N6 medium supplemented with 250mg/l cefotaxime, 50 mg/l hygromycin B) depending on the selection scheme and cultured at 27℃ in dark condition for 3 weeks. Selection was performed with hygromycin B resistance primarily during the first five weeks (callus proliferation stage) after transformation treatment. Selected calli were subcultured 2 weeks after the start of first selection on fresh 2N6-CH medium and further grown for 3 weeks before being transferred to MSRK5SS medium (MS 4.4g, NAA 1mg, Kinetine 5mg, sucrose 20g, sorbitol 30g, proline 0.5g, MES 0.5g, pH 5.8, gelrite 4g, cefotaxium 250mg/l, hygromycine 50mg/l) for second selection and regeneration for 4 weeks at 27℃ under continuous light condition. The regenerated shoots were transferred to MSO medium (MS salts and vitamins, 30g/l sucrose, 2g/l phytagel, pH 5.8) for root induction for 2 weeks before acclimation in a Magenta box containing 0.1% Hyponex solution (Hyponex Co., Imlay City, MI 48444) for 1 week in a culture room. The plantlets were then transplanted to a Wagner pot (1/5000a) in a greenhouse for subsequent growth.

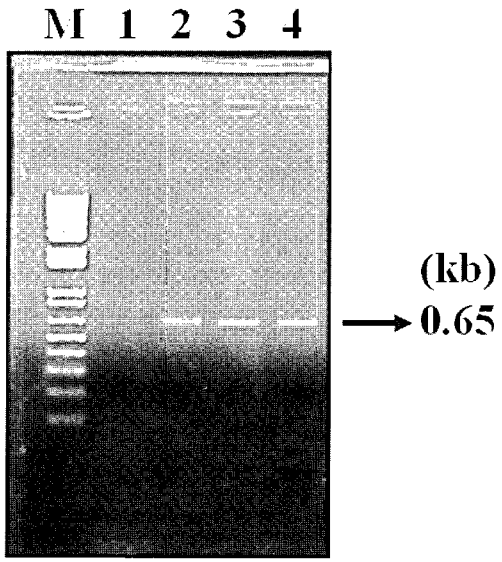

Fig. 2. Electrophoresis pattern of the 650 bp *hpt* gene amplified by PCR using DNA isolated from Ilmibyeo transgenic plants. PCR products of bar or *hpt* gene from T0 plants obtained by first selection scheme (Hyg50).

Plant DNA isolation

The genomic DNA of putative transgenic plant was isolated by the protocol of Doyle and Doyle (1990). About 300mg of young leaf tissue was homogenized in liquid nitrogen and mixed in 900㎕ of preheated (65℃) DNA extraction buffer (0.1M Tris-Cl, 20mM Na_2 ethylenediaminetetraacetic acid (EDTA), 1.4M NaCl and 20% [w/v] hexadecyltrimethylammonium bromide [CTAB] and 0.2% [v/v] β- mercaptoethanol [pH 8.0]) in sterile polypropylene

centrifuge tubes and incubated at 65℃ in a water bath for 1h with occasional gentle swirling. The samples were mixed with chloroform-isoamylalcohol (24:1). The contents were centrifuged at 8800g for 15 min at room temperature, and the aqueous phase was transferred to fresh sterile centrifuge tubes and mixed well with 0.67 vol (600μl) of isopropanol by gently inverting the tubes 5-6 times. This mixture was centrifuged at 8800g for 10 min to pellet the DNA. The pellet was washed with 70% (v/v) ethanol and air-dried. The DNA pellet was dissolved in Tris-EDTA (TE) buffer (pH 8.0).

PCR analysis

The processed DNA samples were used as template to amplify *hpt* gene. The primer sequences were designed for a 650 bp of *hpt* genes. The primer sequences used were as follows: *hpt* forward, 5' - ATCCTTCGCAAGACCCTTCCT-3' ; and *hpt* reverse, 5' - GGTGTCGTCCATCACAGTTTG-3' . PCR was carried out with *Taq* DNA polymerase in 20-μl reaction volume in a thermal cycler (Bio-red com.). The PCR cycle program consisted of initial denaturation at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 1 min 30 sec, followed by a final extension at 72℃ for 7 min. The PCR product was resolved by 1% agarose gel electrophoresis in 0.5 × Tris-acetate-EDTA (TAE) buffer along with 1kb DNA ladder marker (Invitrogen).

Southern blot of transgenic 'rice' plants

All of the transgenic plants were grown under identical conditions. Transgenic plants were analyzed by PCR and Southern hybridization for the integration of the transgene. For Southern blot analysis of transgenic plants, 10μg of purified plant DNA was completely digested with 20 U of *Eco* RI. DNA fragments were resolved on 0.8% agarose gels and blotted on N^+ Hybond nylon membranes. Radiolabeled probe of *HPT gene* sequence was prepared with the ^{32}P.

Results and Discussion

In the PCR analysis of transformation with primers *argE* (Forward & Reverse), no bands were observed both in plantlets regenerated on a medium with 50mg/l of hygromycin only, and in control plantlets. The fragments were observed 650bp in size in selected hygromycin and observed in plantlets transformed and regenerated from callus. In the analysis of transformed plantlets,

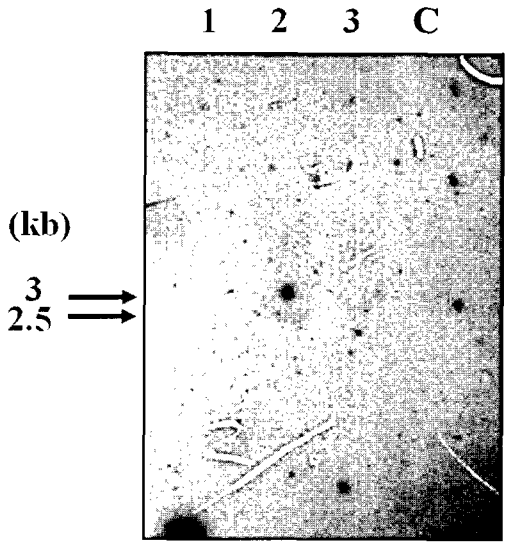

Fig. 3. Southern blot analysis of transgenic rice plants. Genomic DNAs from the leaf tissues of ten independent lines obtained by second selection scheme were digested with *Eco* RI. C, Genomic DNAs from untransformed control plant; 1-3, Genomic DNAs from transgenic rice plants.

DNA fragments, 650 bp in size, were observed in all the transformed plantlets (Fig. 3). Among the plantlets, only in plantlets regenerated from callus and cultured with 50mg/l of hygromycin concentrations where the *hpt* gene was found inserted into the genome. Transformation was confirmed with plantlets cultured only at 50mg/l of hygromycin concentration and regenerated from callus. Based on the results, it can be concluded that 50mg/l of hygromycin concentrations were suitable for callus culture, respectively. In transformation of *rice*, callus culture on a medium with 50mg/l of hygromycin would be useful to increase the successful stable transformation rates.

We can not observed in the fragments of target gene (*argE*). Thus, we examined that selectable marker gene (HPT) inserted in genome of transgenic rice plants. Southern analysis with *hpt* probe confirmed that copy numbers of the selectable gene was introduced into the plant genome (Fig. 3). Some of the transformed plantlet lines except for #3 transgenic lines detected by PCR selected on the medium supplemented with hygromycin did show single band in Southern blot. The assay used for the hygromycin phosphotransferase (HPT) enzyme indicates the effective nature of the selection system. Rice plants regenerated from 40 putative transgenic calli were used for Southern blot analysis (Wu *et al.*, 1997). The *mwti1b* gene in transgenic rice lines was presented 56 plants among 58 hygromycin

resistant rice lines (Mochizuki *et al.*, 1999).

The genetic transformation of rice was accomplished by *Agrobacterium tumefaciens*. A reproducible system for genetic transformation of a Korean rice variety, 'Ilmibyeo' , was developed. Since the transformation system is rapid and obviates the need for establishing long-term callus, there is a strong indication that cell suspension and protoplast regeneration procedure could introduce agronomically useful genes directly to other Korean rice cultivars.

Acknowledgements

This work was supported by a grant from 'Biogreen 21' program of the Rural Development Administration.

Literature Cited

Bent, A.F. 2000. Arabidopsis in planta transformation. Uses, mechanisms, and prospects for transformation of other species. Plant Physiol. 124: 1540-1547.

Cheng, M., J.E. Fry, S. Pang, H. Zhou, C.M. Hironaka, D.R. Duncan, T.W. Conner and Y. Wan. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant J. 115: 971-980.

De Cleene, M. and J. De Ley. 1976. The host range of crown gall. Bot. Rev. 42: 389-466.

Doyle, J.J. and J.I. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Gelvin, S.B. 2003. Agrobacterium-mediated plant transformation: the biology behind the "Gene-Jockeying" tool. Microbiol. Mol. Biol. Rev. 67: 16-37.

Hiei, Y., S. Ohta, T. Komari and T. Kumashiro. 1994. Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271-282.

Hoekema, A., P.R. Hirsch, P.J.J. Hooykaas and R.A. Schilperoort. 1983. A binary vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. Nature 303: 179-180.

Horsch, R.B., R.T. Fraley, S.G. Rogers, P.R. Sanders, A. Lloyd and N. Hoffmann. 1985. A simple and general method of transferring genes into plants. Science 223: 496-498.

Ishida,Y., H. Saito, S. Ohta, Y. Hiei, T. Komari and T. Kumashiro. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. 14: 745-750.

Khanna, H.K. and S.K. Raina. 2002. Elite *indica* transgenic rice plants expressing modified Cry1Ac endotoxin of *Bacillus thuringiensis* show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). Transgenic Res. 11: 411-423.

Lin, Y.J. and Q. Zhang. 2005. Optimizing the tissue culture conditions for high efficiency transformation of *indica* rice. Plant Cell Rep. 23: 540-547.

Mochizuki, A., Y. Nishizawa, H. Onodera, Y. Tabei, S. Toki, Y. Habu, M. Ugaki and Y. Ohashi. 1999. Transgenic rice plants expressing a trypsin inhibitor are resistant against rice stem borers, *Chilo suppressalis*. Entomologia Experimentalis et Applicata 93: 173-178.

Mohanty, A., H. Kathuria, A. Ferjani, A. Sakamoto, P. Mohanty, N. Murata and A.K. Tyagi. 2002. Transgenics of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. Theor. Appl. Genet. 106: 51-57.

Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton and R. Brettell. 1997. *Agrobacterium tumefaciens*-mediated barley transformation. Plant J. 11: 1369-1376.

Wu, C., Y. Fan, C. Zhang, N. Oliva and S. K. Datta. 1997. Transgenic fertile japonica rice plants expressing a modified cryIA(b) gene resistant to yellow stem borer. Plant Cell Rep. 17: 129-132.

Yu, J., J. Wang, W. Lin, S. Li, H. Li, J. Zhou, P. Ni, W. Dong, S. Hu, C. Zeng, J. Zhang , Y. Zhang, R. Li, Z. Xu, S. Li, X. Li, H. Zheng, L. Cong, L. Lin, J. Yin, J. Geng, G. Li, J. Shi, J. Liu, H. Lv, J. Li, J. Wang, Y. Deng, L. Ran, X. Shi, X. Wang, Q. Wu, C. Li, X. Ren, J. Wang, X. Wang, D. Li, D. Liu, X. Zhang, Z. Ji, W. Zhao, Y. Sun, Z. Zhang, J. Bao, Y. Han, L. Dong, J. Ji, P. Chen, S. Wu, J. Liu, Y. Xiao, D. Bu, J. Tan, L. Yang, C. Ye, J. Zhang, J. Xu, Y. Zhou, Y. Yu, B. Zhang, S. Zhuang, H. Wei, B. Liu, M. Lei, H. Yu, Y. Li, H. Xu, S. Wei, X. He, L. Fang, Z. Zhang, Y. Zhang, X. Huang, Z. Su , W. Tong, J. Li, Z. Tong, S. Li, J. Ye, L. Wang, L. Fang, T. Lei, C. Chen, H. Chen, Z. Xu, H. Li, H. Huang, F. Zhang, H. Xu, N. Li, C. Zhao, S. Li, L. Dong, Y. Huang, L. Li, Y. Xi, Q. Qi, W. Li, B. Zhang, W. Hu, Y. Zhang, X. Tian, Y. Jiao, X. Liang, J. Jin, L. Gao, W. Zheng, B. Hao, S. Liu, W. Wang, L. Yuan, M. Cao, J. McDermott, R. Samudrala, J. Wang, G.K. Wong and H. Yang. 2005. The genomes of *Oryza sativa*: a history of duplications. PLoS. Biol. 3: e38.

Zhang, J. Q. Feng, C. Jin, D. Qiu, L. Zhang, K. Xie, D. Yuan, B. Han, Q. Zhang and S. Wang. 2005. Features of the expressed sequences revealed by a large-scale analysis of ESTs from a normalized cDNA library of the elite *indica* rice cultivar Minghui 63. Plant J. 42: 772-780.

(Received 23 May 2007 ; Accepted 12 June 2007)