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Generation of Transgenic Rice without Antibiotic Selection Marker through *Agrobacterium*-mediated Co-transformation System

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Development of transgenic plant increasing crop yield or disease resistance is good way to solve the world food shortage. However, the persistence of marker genes in crops leads to serious public concerns about the safety of transgenic crops. In the present paper, we developed marker-free transgenic rice inserted high molecular-weight glutenin subunit (HMW-GS) gene (*Dx*5) from the Korean wheat cultivar 'Jokyeong' using *Agrobacterium*-mediated co-transformation method. Two expression cassettes comprised of separate DNA fragments containing only the *Dx*5 and hygromycin resistance (*HPTII*) genes were introduced separately into *Agrobacterium tumefaciens* EHA105 strain for co-infection. Each EHA105 strain harboring *Dx*5 or *HPTII* was infected into rice calli at a 3: 1 ratio of EHA105 with *Dx*5 gene and EHA105 with *HPTII* gene expressing cassette. Then, among 66 hygromycin-resistant transformants, we obtained two transgenic lines inserted with both the *Dx*5 and *HPTII* genes into the rice genome. We reconfirmed integration of the *Dx*5 and *HPTII* genes into the rice genome by Southern blot analysis. Wheat *Dx*5 transcripts in T_1 rice seeds were examined with semi-quantitative RT-PCR. Finally, the marker-free plants containing only the *Dx*5 gene were successfully screened at the T_1 generation. These results show that a co-infection system with two expression cassettes could be an efficient strategy to generate marker-free transgenic rice plants.

Key words : *Agrobacterium*-mediated co-transformation, high-molecular-weight glutenin subunit (HMW-GS) gene, hygromycin (*HPTII*) resistance, marker-free transgenic rice, two expression cassettes

Introduction

Rice is predominant staple food with wheat in the world and it provides more than 23% of the world's dietary energy supply. Rice is also a good source of thiamine, riboflavin and dietary fibre[18]. During the last 40 years, the technological advances for conventional breeding led to the development of high-yielding and disease resistant varieties of rice in world food production. However, Population in the world is currently growing at a rate of around 1.10% per year and it is estimated that it will become 8.5 billion by 2025 [1]. Forty % of more rice production by 2030 was demanded to satisfy the growing demand. And the agricultural environment conditions have been recently changing due to global temperature warming. However, there may be a way to overcome this issue through genetic engineering with conventional breeding [1].

Antibiotic genes such as *hygromycin phosphotransferase II* (*HPTII*) have been used as selectable markers in a part of the plant transformation process to select transgenic cells from which intact transgenic plants can be regenerated [10]. The *bar* gene which was herbicide resistance from *Streptomycies hygroscopicus* detoxifies the herbicide glufosinate. This gene has been widely used as a selectable marker in plant transformation systems. Even though several risk-assessment reports have shown that the selectable marker genes and their products do not alter the reproductive biology or potential for gene flow and do not increase the risks for adverse effects to other organisms, the persistence of selectable marker genes in crops destined for field cultivation and human food leads to serious public concerns about the safety of transgenic crops [14,17].

Several genetic engineering techniques such as Cre-*lox*P system were developed to generate marker free transgenic

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plant. The Cre-*lox*P recombination system of bacteriophage PI needs expression of the Cre protein which cause the recombination between *loxP* sites. The recombination between *loxP* sites results in excision and circularization of intervening DNA. However, the Cre-*lox*P recombination system requires either the transient expression of *cre* gene, crossing with a recombinase-expressing line, or an inducible factor to turn on the expression of *cre* gene [3]. Multi-Auto-Transformation (MAT) vector system was also developed to select marker-free transgenic plants. In MAT system, the oncogenes of Agrobacterium such as ipt, iaaM/H, and rol control the endogenous levels of plant hormones and regenerate transgenic plants, and then are removed by the R/RS system [12]. Co-transformation is a method for production of marker free transformations based on *Agrobacterium*-mediated transformation. For this strategy, three transformation approaches were developed: introduction of two *T-DNAs* in separate *Agrobacterium* strains, introduction of two *T-DNA* carried by different replicons within the same *Agrobacterium* strain, and introduction of two *T-DNAs* located on the same replicon within an *Agrobacterium* strain[8]. In this study, we modified the *pCAMBIA*1300 vector for introduction of target gene and generated the marker free transgenic rice through the co-transformation system with two *Agrobacterium* cells.

Materials and Methods

Cloning of the wheat *Dx*5 glutenin gene

'Jokyeong' (*Triticum aestivum* L. cv. Jokyeong) was used for cloning the *Dx*5 glutenin gene. The *Dx*5 gene was amplified by polymerase chain reaction (PCR) of genomic DNA using the primers Dx5-F (primer sequences: 5'-AGGG TACCGAGATGGCTAAGC GGTTAG-3') and Dx5-R (primer sequences: 5'-GATCTAGATCACTGGCTGGCCGAC AATG-3'), which were designed from a GenBank sequence (accession no. X12928). The PCR temperature cycling conditions were 4 min at 94°C, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min, and a final extension at 72°C for 10 min. The amplified products were separated on a 1% agarose gel and visualized with ethidium bromide. The amplified products were sub-cloned using a TOPO TA Cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA).

DNA constructs

To make a marker-free vector, we first inserted *GluB1* promoter, which regulates seed storage protein genes in the endosperm of rice seeds [21] into the to the *pCAMBIA*1300 binary vector. The *HPTII* expression cassette (*CaMV 35S* promoter-*HPTII* gene-*CaMV 35S* terminator) in the *pCAMBIA*1300 binary vector was removed by *Xho*I and *Eco*RI restriction enzyme treatment. After klenow enzyme treatment for blunt ligation, the vector was self-ligated. Then, amplified the *Dx*5 gene with the *Eco*RI and *Kpn*I restriction enzyme sites was constructed into modified *pCAMBIA*1300 binary vectors under the control of *GluB1* promoter. The positive selectable marker cassette for co-transformation used an empty *pCAMBIA*1300 binary vector (Fig. 1B).

Agrobacterium handling

Agrobacterium tumefaciens EHA105 was transformed with *Dx*5 cloned binary vectors and an empty vector containing *HPTII* for the selectable marker using the freeze-thaw method [4]. Transformants were selected on YEP media containing kanamycin (50 mg/l). Transformation was confirmed by a restriction analysis and PCR amplification of plasmids mini-prepped from each *Agrobacterium* strain [2].

Rice co-transformation

Mature seeds of *Oryza sativa* L. subsp. *japonica* var. 'Dongjin' were used to induce callus formation on callus induction (CI) medium (N_6 salts [6] with vitamins, 2.5 g/l proline, 2 mg/l 2,4-D, 0.5 g/l casamino acid, 30 g/l sucrose and 2 g/l gelrite, pH 5.7). After 21 days incubation in the dark at 25°C, the scutellum-derived calli were excised and preincubated on CI medium for 1 week. *Agrobacterium* was grown for 2 days on YEP solid medium containing antibiotics at 25°C. Agrobacterial cells were resuspended in suspension medium (N6 salts with vitamins, 2 mg/l 2,4-D, 0.5 g/l casamino acid, 30 g/l sucrose, and 10 g/l glucose, pH 5.7) with 200 μM acetosyringone as a final concentration. After two *Agrobacterium* cells were mixed in a 3:1 ratio of

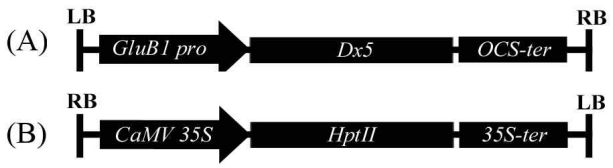

Fig. 1. Vector constructs expressing the *Dx*5 (A) and hygromycin phosphotransferase II (B) genes in binary vectors. *GluB1 pro*, glutelin B1 promoter; *OCS-ter*, octopine synthase terminator; *CaMV 35S*, cauliflower mosaic virus promoter; *35S-ter*, 35S terminator; RB, right border; LB, left border.

EHA105 with *Dx*5 gene expressing cassette and EHA105 with *HPTII* gene expressing cassette, the calli were transformed by swirling in a mixture of *Agrobacterium* cultures for 30 min. The calli were blotted on Whatman no. 1 paper and cocultivated on cocultivation medium (N_6 salts with vitamins, 2 mg/l 2,4-D, 0.5 g/l casamino acid, 30 g/l sucrose, 10 g/l glucose, and 2 g/l gelrite, pH 5.2 with 200 μM acetosyringone as a final concentration). After 3 days, the calli were washed with liquid CI medium supplemented with 250 mg/l cefotaxime and 150 mg/l timentin and placed on the selection medium (CI medium supplemented with 50 mg/l hygromycin, 250 mg/l cefotaxime). After selection and regeneration, the regenerated plantlets were acclimatized and grown in a greenhouse.

PCR analyses of transformants

PCR was performed with the GeneAmp System 9700 (Applied Biosystems, Foster City, CA, USA) with a gene-specific primer set (*Dx*5; forward 5′-GGGACAATACGAGC AGCAAA-3′, reverse 5′-CTTGTTCCGGTT GTTGCCAT-3′, *HPTII*; forward 5′-CGCTTCTGCGGGCGATTT-3′, reverse 5′-CCCATTCGGACCGCAAGGA-3′) and EF Taq DNA polymerase (Solgent Co. Seoul, South Korea). Each reaction mixture (30 μM) consisted of 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 40 mM KCl, 250 μM dNTPs, and 1 U Taq DNA polymerase. Amplified products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized with an ultraviolet illuminator.

Southern hybridization analysis

Rice genomic DNA was prepared using the CTAB extraction method [19]. Aliquots of 5 μg of purified DNA were digested with restriction endonuclease (*EcoR*I), size-fractionated on a 0.8% agarose gel, and the DNA was transferred to a nylon membrane through capillary blotting in 10× SSC (Gene Screen, DuPont, Wilmington, DE, USA). The blots were labeled using AlkPhos Direct (Amersham, GE Healthcare. Piscataway, NJ, USA) according to the manufacturer's instructions. After hybridization, the filters were washed for 30 min at 55°C to remove unlabelled probe. Subsequently, CD-star Detection Reagent (Amersham, GE Healthcare) was used to detect and generate signals.

RNA extraction and RT-PCR analysis

T_1 generation seeds were frozen in liquid nitrogen and then ground to powder using a mortar and pestle. Total RNA was extracted using a method reported previously [15]. The isolated RNA preparations were reverse-transcribed with oligo-dT primer and a First Strand cDNA Synthesis kit for RT-PCR (Roche Co., Basel, Switzerland) with gene-specific primers. The primers were as follows: Dx5 forward 5′-GGGACAATACGAGCAGCAAA-3′, *Dx*5 reverse 5′-CTT GTTCCGGTTGTTGCCAT-3′; *OsActin* primers were used as internal standards for mRNA expression profiling[16,20]. The PCR conditions consisted of initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The experiments were repeated three times, and all produced similar results. The *OsActin* control primers were 5′-GGAACTGGTATGGTCAAGGC-3′ and 5′-AGTCTCATG GATACCCGCAG-3′ [5].

Results

Vector construction and *Agrobacterium* transformation for marker-free transgenic rice

Co-transformation experiments were conducted using two expression cassettes containing separate linear DNA fragments with the *Dx*5 and hygromycin resistance (*HPTII*) genes. The marker-free vector cassette was constructed with the modified *pCAMBIA*1300, which was changed for the *GluB1* promoter after removing the cauliflower mosaic virus promoter (*CaMV35S*) (Fig. 1A). The positive selectable marker cassette for co-transformation used an empty *pCAMBIA*1300 vector (Fig. 1B). The *HPTII* coding sequence was under the control of *CaMV35S* and was followed by the *OCS* termination sequence from *Agrobacterium tumefaciens*; HMW glutenin subunit *Dx*5 coding sequences were flanked by endosperm-specific *GluB1* promoters from rice. The two expression binary vectors were separately introduced into the *A. tumefaciens* EHA105 strain for plant transformation. Each binary vector was rescued from the EHA105 strain harboring *HPTII* and *Dx*5, and the *HPTII* and *Dx*5 genes were validated by PCR analysis.

Generation of marker-free *Dx*5 transgenic rice plant

Each EHA105 strain harboring *Dx*5 expression vector or *HPTII* expression vector was cultured in YEP medium for plant transformation. The cultured cells were resuspended to OD_{600}=0.1 in AAM medium [11], and each *Dx*5 and *HPTII* cell was added at a 3: 1 ratio. These mixed cells were co-infected into rice calli. The transformed calli were selected

with hygromycin because we co-infected calli with the *HPTII* gene. We obtained 66 independent hygromycin-resistant transformants through co-infection in the *Agrobacterium* transformation system.

Genomic DNA from 66 independent T_0 transformants was extracted and insertion of the *HPTII* and *Dx*5 genes was analyzed by PCR with gene specific primers. As shown in Fig. 2, the *HPTII* gene was amplified in all transformants, but no PCR products of 'Jokyeong' and 'Dongjin' (negative controls) were detected. Next, we investigated insertion of the *Dx*5 gene into the rice genome within transformants by PCR analysis. Among 66 independent transgenic lines, the *Dx*5 gene was amplified as a PCR product in only two transformants (2 and 7 lines), which was the same PCR product size of Jokyeong and the plasmid used as positive controls (Fig. 2). This result indicates that only two transgenic lines harbored both the *Dx*5 and *HPTII* genes. The co-transformation frequency was about 3% in our experimental conditions (Table 1).

We performed Southern blot analysis with the *Dx*5 and *HPTII* genes as probes to validate their insertion and guess segregation ratio of the marker-free plant in T_1 plants. Two signal bands in both selected transformants lines (2 and 7 lines) were detected when we used the *HPTII* gene as a probe; however, two integrations of the *Dx*5 gene occurred in transformant line 2 and a single integration was found in transformant line 7 (Fig. 3). To examine whether these two transformants were different, we analyzed the insertion position of the *Dx*5 gene in the two transformants by flanking *T-DNA* sequencing analysis. The *Dx*5 integration position of the line 2 was in chromosomes 2 and 8, the *Dx*5 integration position of the line 7 was in chromosomes 10 (data not shown). These results indicating that even though the *HPTII* gene was integrated into the rice genome in a similar pattern, transformant lines were independent transgenic rice plants.

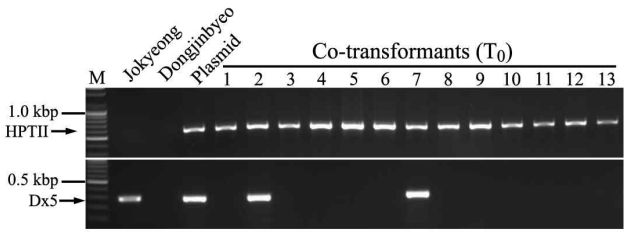

Fig. 2. Identification of co-transformants by gene specific primer sets. *HPTII* (upper panel) and *Dx*5 (lower panel) genes were amplified using *HPTII* and *Dx*5 specific primer sets, respectively. M, molecular marker; Jokyeong, Korean wheat cultivar; Dongjinbyeo (Korean rice cultivar), non-transgenic plant; plasmid, vector construct; 1-13, co-transformant transgenic lines. Genomic DNAs from each plant were used as the template for *Dx*5 and *HPTII* specific amplification. The reaction products of the sample plant were analyzed by electrophoresis on a 1.0% agarose gel.

Dx5 gene expression analysis in the co-transformed rice plants

Because *Dx*5 expression in rice endosperm is important for rice flour quality and we used the GluB1 promoter to express *Dx*5, total RNA from randomly selected T_1 generation transgenic seeds (lines 2 and 7) was extracted, and *Dx*5 gene transcript level was examined by semi-quantitative

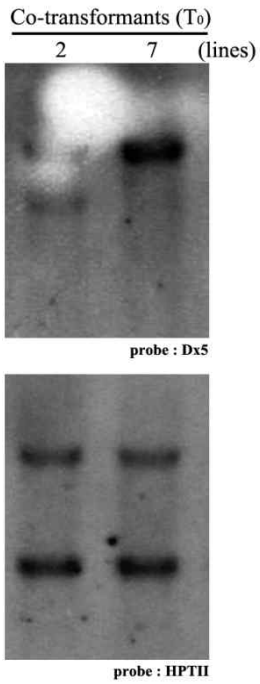

Fig. 3. Southern hybridization analysis of *Dx*5 (upper panel) and *HPTII* (lower panel) genes from T_0 co-transformants. The 0.28 kb (*Dx*5) and 0.54 kb (*HPTII*) fragments were amplified by PCR using specific primer sets as the probe.

Table 1. Co-transformation efficiency calculated during regeneration in rice transformation experiments

Gene	No. of transformants	No. of plants containing the *Dx*5	Frequency of co-transformation (%)
Dx5	66	2	3.0

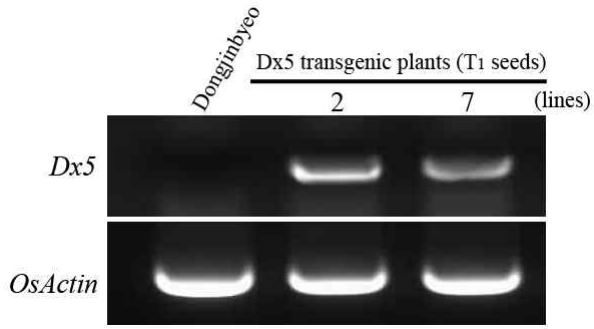

Fig. 4. Expression analysis of the *Dx*5 gene from T_1 seeds. RT-PCR was performed with *Dx*5 T_1 seed transcripts to measure *Dx*5 mRNA expression. Gene-specific PCR primers (forward and reverse primers) were designed to amplify the *Dx*5 gene. *OsActin* was used as the control. The amplified products were analyzed by electrophoresis on a 1.0% agarose gel.

RT-PCR. The *Dx*5 transcripts were successively expressed in the T_1 generation transgenic seeds (lines 2 and 7), whereas *Dx*5 expression in 'Dongjin' was not detected (Fig. 4). *OsActin* expression was used as a quantitative control.

Selection of marker-free plants harboring the *Dx*5 gene at the T_1 generation

To select *Dx*5 marker-free plants harboring only the *Dx*5 gene, 72 T_1 generation seeds of the transformant line 2 were planted in soil and genomic DNA was extracted from leaves of plantlets after 4 weeks. Insertion of the *Dx*5 and *HPTII* genes was investigated by PCR analysis. As shown in Fig. 5, most of the transgenic lines harbored both the *Dx*5 and *HPTII* genes, and some inserted only the *HPTII* gene. However, transgenic 3 and 14 lines contained only the *Dx*5 gene (Fig. 5). This result shows that marker-free plants containing only the *Dx*5 gene were successfully screened at the T_1 generation and that co-infection using the *Agrobacterium* transformation system was an efficient strategy to produce marker-free transgenic rice plants.

Discussion

Generation of marker-free transgenic plant is important way to solve the public concerns about the safety of antibiotics and herbicides [8]. In this paper, we used the *Agrobacterium*-mediated co-transformation method with two expression cassettes comprised of separate DNA fragments containing the *Dx*5 and *HPTII* resistance genes to produce transgenic rice plants free of herbicide and antibiotic resistance genes (Fig. 1). This method is one way to separate selectable marker genes from transgenes at the transformation stage. *Agrobacterium*-mediated transformation is usually used for this purpose, because separate integration events occur more regularly using this method than with direct gene delivery methods. A wide range of variations have been reported, and it is difficult to provide a comprehensive evaluation on the general applicability of the different methods [8]. However, co-transformation frequencies are higher than expected for independent events. The co-transformation frequency in our experimental conditions was 3% (Table 1). In a previous report, co-transformation frequency in rice was about from 2% to 14%[13]. This result indicates that transformation efficiency is dependent on rice cultivar and the experimental conditions.

Several research groups tried to develop transgenic plant with one *T-DNA*, which without antibiotic or herbicide marker genes, and then screened transformants harboring transgene by PCR analysis with genomic DNA [9]. This approach needs a considerable time and physical labor, and the efficiency of screening of insertion of *T-DNA* is lower. The Cre-*lox*P system was developed to remove target gene by cleavage of site-specific DNA fragment from genomic DNA [7]. However, this approach requires another insertion of *cre* gene and there is concern about another recombination. In co-transformation approach, because selectable marker gene can subsequently be removed from the

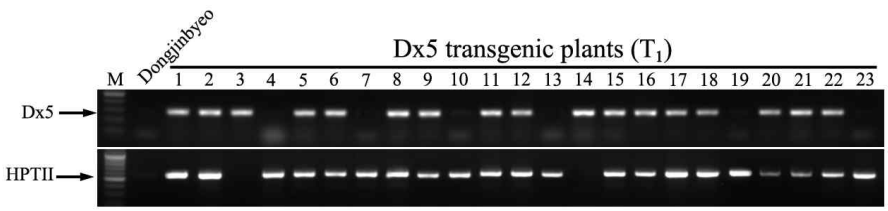

Fig. 5. PCR analysis of T_1 progeny (line 2 in Fig. 2) to select marker-free transgenic plants containing only the *Dx*5 gene. M, molecular marker; Dongjinbyeo, non-transgenic plant as negative control; 1-23, T_1 progeny lines from T_0 co-transformants containing both the *Dx*5 and *HPTII* genes. The reaction products were analyzed by electrophoresis on a 1.0% agarose gel.

plant genome during segregation and recombination that occurs during sexual reproduction, there is no imprecise excision.

Wheat high molecule-weight glutelin subunits (HMW-GSs) are major determinants for bread quality such as gluten elasticity [22]. HMW-GSs are specifically found in the wheat, not rice. We used the *Dx*5 gene, which is one of HMW-GS gene, and validated the insertion of the *Dx*5 gene in transformants through PCR analysis with gene specific primers and Southern blot analysis (Fig. 2, 3). The *GluB1* promoter derived from a glutelin gene can express the high-level foreign gene in monocot such as rice and barley[23]. The *Dx*5 transcripts in the marker-free transgenic lines were stably expressed in T_1 generation rice seeds (Fig. 4). Finally, we obtained two or three marker-free plants containing only the *Dx*5 gene through *Agrobacterium*-mediated co-transformation at the T_1 generation stage of the transformant lines (2 and 7 lines). According to flanking *T-DNA* sequencing and Southern blot analysis, The *Dx*5 integration position of the two transformant lines was different. And the *Dx*5 gene transcripts of line 2 were slightly higher than line 7 (Fig. 4). These results indicating that line 2 and 7 were revealed as independent lines. The morphological characteristics of the marker-fee transgenic rice expressing the *Dx*5 gene were similar with that of 'Dongjin' (data not shown). Our marker-fee transgenic rice expressing the *Dx*5 gene in this study is first trial using *Agrobacterium*-mediated co-transformation in the world.

Taken together, although generating marker-free plants based on *Agrobacterium*-mediated co-transformation using two different expression cassettes was more time consuming, this method was an efficient strategy to produced marker-free transgenic rice plants.

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초록 : 아그로박테리움 동시 형질전환 시스템을 통한 항생제 선발 마커가 없는 형질전환벼의 생산

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작물의 수확량이나 병 저항성을 증가시키는 형질전환 식물체 개발은 세계 식량 부족을 해결하는 좋은 방법이다. 하지만 항생제나 제초제의 사용은 형질전환 작물의 안전에 대해서 일반 사람들의 심각한 우려를 초래한다. 본 연구에서는, 아그로박테리움을 이용한 동시 형질전환 방법을 이용하여 한국의 밀 재배종인 '조경밀'의 유전자인, 고분자 글루테닌 서브유닛[high molecular-weight glutenin subunit (HMW-GS)] Dx5가 삽입된 마커프리 형질전환벼를 개발하였다. 각각 Dx5 유전자와 하이그로마이신(HPTII) 저항성 유전자만으로 구성된 두 종류의 발현 카셋트(Two expression cassettes)를 독립적으로 아그로박테리움 EHA105에 도입하였고, Dx5와 HPTII가 도입된 각각의 EHA105 아그로박테리움을 3:1 비율로 혼합하여 벼 캘러스에 접종하였다. 66개의 HPTII 저항성 형질전환체 중에서 벼 게놈에 Dx5와 HPTII가 모두 삽입된 2개의 형질전환 라인을 획득하였다. Dx5와 HPTII가 벼 게놈에 도입된 것을 Southern blot을 통해서 다시 확인하였다. 또한, semi-quantitative RT-PCR을 통해 형질전환벼 T_1 세대 종자의 밀 Dx5 전사여부를 확인하였고 결국, Dx5 유전자만을 가지는 마커프리 형질전환벼를 T_1 세대에서 선발할 수 있었다. 본 연구 결과는 두 종류의 발현 카셋트를 사용한 아그로박테리움 동시 접종 시스템이 마커프리 형질전환벼를 생산하기 위한 효과적인 전략이 될 수 있음을 보여준다.