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Development of Marker-free Transgenic Rice Expressing the Wheat Storage Protein, Glu-1Dy10, for Increasing Quality Processing of Bread and Noodles

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Rice flour is used in many food products. However, dough made from rice lacks extensibility and elasticity, making it less suitable than wheat for many food products such as bread and noodles. The high-molecular weight glutenin subunits (HMW-GS) of wheat play a crucial role in determining the processing properties of the wheat grain. This paper describes the development of marker-free transgenic rice plants expressing a wheat Glu-Dy10 gene encoding the HMG-GS from the Korean wheat cultivar 'Jokyeong' using *Agrobacterium*-mediated co-transformation. Two expression cassettes, consisting of separate DNA fragments containing *Glu-1Dy10* and hygromycin phosphotransferase II (*HPTII*) resistance genes, were introduced separately into *Agrobacterium tumefaciens* EHA105 for co-infection. Each EHA105 strain harboring *Glu-1Dy10* or *HPTII* was infected into rice calli at a 3: 1 ratio of *Glu-1Bx7* and *HPTII*. Among 290 hygromycin-resistant T_0 plants, we obtained 29 transgenic lines with both the *Glu-1Dy10* and *HPTII* genes inserted into the rice genome. We reconfirmed the integration of the *Glu-1Dy10* gene into the rice genome by Southern blot analysis. Transcripts and proteins of the *Glu-1Dy10* in transgenic rice seeds were examined by semi-quantitative RT-PCR and Western blot analysis. The marker-free plants containing only the *Glu-1Dy10* gene were successfully screened in the T_1 generation.

Key words : Co-transformation, high-molecular-weight glutenin subunit (HMW-GS) protein, marker-free transgenic rice, wheat

Introduction

Improvement of rice quality, especially that of rice flour is of great relevance to many Asian counties, where rice is widely cultivated, and rice flour has been used for many food products. However, dough made from rice lacks extensibility and elasticity. A probable cause is lack of proteins responsible for this trait in the rice endosperm. On the other hand, dough made from wheat flour is elastic and extensible making it suitable for many food products particularly for bread [26]. Transgenic rice with improved dough functionality by transformation of wheat gluten genes, such as high and low-molecular weight glutenin subunits, gliadins maybe substitute or complement wheat flour for making bread.

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Wheat flour is different from other cereal flours, including rice. The unique processing properties of wheat flour result from the unusual biomechanical properties of the gluten proteins, which form a network conferring elasticity and extensibility to the dough [5]. Gluten proteins consist of monomeric gliadins and polymeric glutenins. Gliadins are single chain molecules which form only intra-chain disulphide bonds. In contrast, the glutenin subunits form both inter- and intra-chain disulphide bonds. The high molecular weight glutenin subunits (HMW-GS) of wheat play an important role in determining the functional properties of wheat dough [19, 20, 27, 29].

Bread wheat contains from three to six HMW-GS genes, with tightly linked pairs of genes encoding x- and y-type subunits being present at each of the Glu-A1, Glu-B1, and Glu-D1 loci on the long arms of chromosomes 1A, 1B, and 1D, respectively [21]. Allelic differences in the HMW-GS composition result in effects on the structures and properties of the glutenin polymers and hence on bread-making quality [22, 28].

Glu-1Dy10, one of the HMW-GSs in the D1 chromosome, consists of 648 amino acids and five cysteine residues in the

C-terminal domain. The central domain has an additional cysteine residue that is present close to the end of the C-terminus in the y-type subunits and, occasionally, closes to the N-terminus in the x-type subunits. The y-type subunits are highly cross-linked with x-type subunits and enhance mixing strength and tolerance of dough. In particular, HMW-GS 1Ax1 and 1Dx5+1Dy10, encoded by chromosomes 1A and 1D, respectively, are associated with strong dough and good bread-making quality.

Several HMW-GS genes have been shown to be functional when transformed into *Escherichia coli* [10], tobacco [23], wheat [1, 2, 5, 6] and tritordeum [24].

The genetic engineering of transgenic plants in most crop species requires the use of selectable marker genes and selective agents, such as herbicides and antibiotics in order to minimize regeneration of non-transformed tissues. However, the presence of selectable marker genes in transgenic crops destined for field cultivation and human food leads to serious public concerns about the safety of transgenic crops, even though several risk-assessment reports [14, 25] have shown that neither the genes nor their products are harmful to human or environmental health. Because combination of wheat gluten genes is important to make transgenic rice plants with good bread-making quality, transgene pyramiding of transgenic rice plants, which containing various wheat gluten genes is required. Repeated use of the same promoter and a polyadenylation signal for different selectable marker genes could result in transcriptional gene silencing [12]. Therefore, eliminating selectable marker genes is crucial for stacking multiple traits in a transgenic plant. Moreover, generating marker-free transgenic plants responds not only to public concerns over the safety of genetically engineered crops, but supports multiple transformation cycles for transgene pyramiding.

In this study, we produced marker-free transgenic rice expressing the wheat HMW-GS protein, Glu-1Dy10, without any herbicide or antibiotic resistance marker genes. The marker-free transgenic plant expressing *Glu-1Dy10* gene is critical material for generating transgenic plant advanced quality processing of bread and noodle without antibiotic markers.

Materials and Methods

Cloning of the wheat *Glu-1Dy10* glutenin gene

'Jokyeong' (*Triticum aestivum* L. cv. Jokyeong) was used for cloning the *Glu-1Dy10* gluenin gene. The *Glu-1Dy10* gene was amplified by polymerase chain reaction (PCR) of genomic DNA using the primers *Glu-1Dy10-CF (primer sequences: 5*′- AGGGTACCGAGATGGCTAAGCGGCTGG - 3′) and *Glu-1Dy10-CR* (primer sequences: 5′ - GATCTAGATCACTGGCTAGCCGACAATG -3′), which were designed from a sequence on GenBank (accession no. X12929). The PCR temperature cycling conditions were 4 min at 94°C, followed by 35 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 amplification products were separated on a 1% agarose gel and visualized with EtBr. 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 amplified promoter region of high-molecular weight glutenin gene (Genbank accession no. AY795083.1) from wheat cultivar 'Jokyeong'. And then amplified promoter was inserted into the *pBTEX* binary vector, which modified from *pCAMBIA1300* binary vector. The *HPTII* expression cassette (*CaMV 35S* promoter-*HPTII* gene-*CaMV 35S* terminator) in the *pBTEX* binary vector was removed by *XhoI* and *EcoRI* restriction enzyme treatment. After klenow enzyme treatment for blunt ligation, the vector was self-ligated. Then, amplified the *Glu-1Dy10* gene with the *KpnI* and *XbaI* restriction enzyme sites was constructed into *pBTEX* binary vectors under the control of HMW (high-molecular weight) glutenin promoter (Fig. 1, upper panel). The positive selectable marker cassette for co-transformation was used by an empty *pBTEX* binary vector (Fig. 1, lower panel).

Agrobacterium handling

Competent *Agrobacterium tumefaciens* EHA105 was transformed with *Glu-1Dy10*- cloned binary vector and an empty vector containing *HPTII* for the selectable marker using the freeze-thaw method [7]. T_0 plants were selected on YEP media containing kanamycin (50 mg/l). Transformation was confirmed by PCR amplification of plasmids mini-prepped from each *Agrobacterium* strain [3].

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 [9] 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 of incubation in the dark at 25°C, the scutellum-derived calli were excised and preincubated on CI medium for 1 week. Agrobacterial cells were grown on YEP solid medium containing antibiotics at 25°C for 2 days. And then, agrobacterial cells were resuspended in suspension medium (N_6 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 EHA105 with *Glu-1Dy10* gene expressing cassette and EHA105 with *HPTII* gene expressing cassette, the calli were transformed by swirling in the mixture of *Agrobacterium* cultures for 30 min. The calli were blotted on Whatman no. 1 paper and cocultivated on the 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 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 analysis of T_0 plants

PCR was performed with the GeneAmp System 9700 (Applied Biosystems, Foster City, CA, USA) with a gene-specific primer set (*Glu-1Dy10*; forward 5′-CGCAAGACAATATGAGCAAAC-3′, reverse 5′-GTTGCCTTTGTCCTGTGTGCT-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 EtBr, and visualized with a UV illuminator.

Southern hybridization analysis

Rice genomic DNA was prepared by the CTAB extraction method [30]. Aliquots of 5 μg of purified DNA were digested with restriction endonuclease (*EcoRI*), 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. Piscataway, NJ, USA) 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 [33]. The isolated RNA preparations were then 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: *Glu-1Dy10* forward 5′-CGCAAGACAATATGAGCAAAC-3′, *Glu-1Dy10* reverse 5′-GTTGCCTTTGTCCTGTGTGCT -3′; *OsActin* primers were used as internal standards for mRNA expression profiling [17, 32]. 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 2 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′- AGTCTCATGGATACCCGCAG -3′ [8].

Protein extraction and Western blot

T_1 generation seeds were frozen in liquid nitrogen and then ground to powder using a mortar and pestle. Total storage proteins in the rice endosperm were extracted with 50mM Tris-HCl (pH 8.0) containing 2% SDS, 50% of 1-propanol and 1% of dithiothreitol, as described [4]. Amount of extracted total proteins was measured by Nanodrop Spectrophotometer (ND-1000, Thermo Fischer Scientific, Wilmington DE, USA). Western blot analysis was performed as described [18].

Results and Discussion

DNA 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 *Glu-1Dy10* and hygromycin resistance

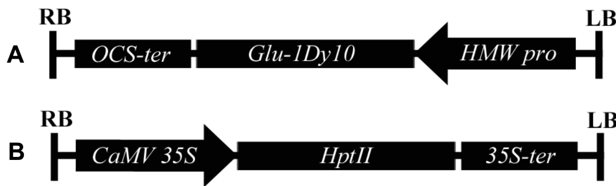

Fig. 1. Vector constructs expressing the *Glu-1Dy10* (upper panel) and hygromycin phosphotransferase II (*HPTII*) (lower panel) genes in the binary vectors. HMW pro, high-molecular weight promoter; OCS-ter, octopine synthase terminator; CaMV 35S, cauliflower mosaic virus promoter; 35S-ter, 35S terminator; RB, right border; LB, left border.

(*HPTII*) genes. The amplified *Glu-1Dy10* gene from genomic DNA of *Triticum aestivum* cv. Jokyeong was constructed into the *pBTEX* marker-free vector, which was changed with the HMW promoter after removing hygromycin resistance gene (*HPTII*) and the cauliflower mosaic virus promoter (*CaMV35S*) (Fig. 1A, upper panel). An empty *pBTEX* vector was used as the positive selectable marker cassette for co-transformation (Fig. 1B, lower panel). The two expression binary vectors were separately introduced into *A. tumefaciens* EHA105 strain for plant transformation. Each binary vector was rescued from the EHA105 strain harboring *Glu-1Dy10* and the *HPTII*, and then the *HPTII* and *Glu-1Dy10* genes were validated by PCR analysis with specific primers.

Generation of marker-free *Glu-1Dy10* transgenic rice plants

Each EHA105 strain harboring *Glu-1Dy10* 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 *Glu-1Dy10* and *HPTII* cell was added at a 3:1 ratio. These mixed cells were co-infected into rice calli. The transformed calli were selected in hygromycin medium because we co-infected EHA 105 cells containing *HPTII* gene to calli. We obtained 290 independent hygromycin-resistant T_0 plants through *Agrobacterium*-mediated co-transformation system. Genomic DNA from 290 independent T_0 plants was extracted and insertion of *HPTII* and *Glu-1Dy10* genes was analyzed using PCR analysis with gene specific primers. As shown in Figure 2, *HPTII* gene in all of T_0 plants was amplified, but no PCR products in 'Dongjin' used as negative controls were detected. Next, we investigate the insertion of *Glu-1Dy10* gene in T_0 plants. Among 290 independent transgenic lines, only 29 T_0 plants contained *Glu-1Dy10* gene (Fig. 2). This result means that 29 transgenic lines harbored both *Glu-1Dy10* and *HPTII* genes. And the co-transformation frequency was 10% in our experimental system (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. Although the generating of marker-free plants based on the *Agrobacterium*-mediated co-trans-

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

Gene	No. of T_0 plants	No. of plants containing the *Glu-1Dy10*	Frequency of co-transformation (%)
Glu-1Dy10	290	29	10.0

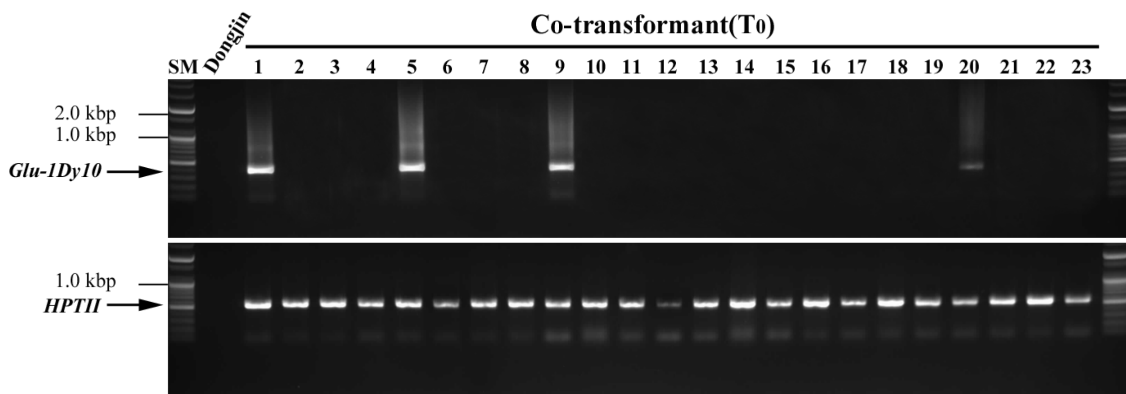

Fig. 2. Identification of T_0 plants by gene specific primer sets. *Glu-1Dy10* (upper panel) and *HPTII* (lower panel) genes were amplified using *Glu-1Dy10* and *HPTII* specific primer sets, respectively. SM, molecular marker; 'Dongjin' (Korean rice cultivar), non-transgenic plant; 1-23, co-transformed transgenic lines. Genomic DNAs from each plant were used as the template for *Glu-1Dy10* and *HPTII* specific amplification. The reaction products of the sample plant were analyzed by electrophoresis on a 1.0% agarose gel.

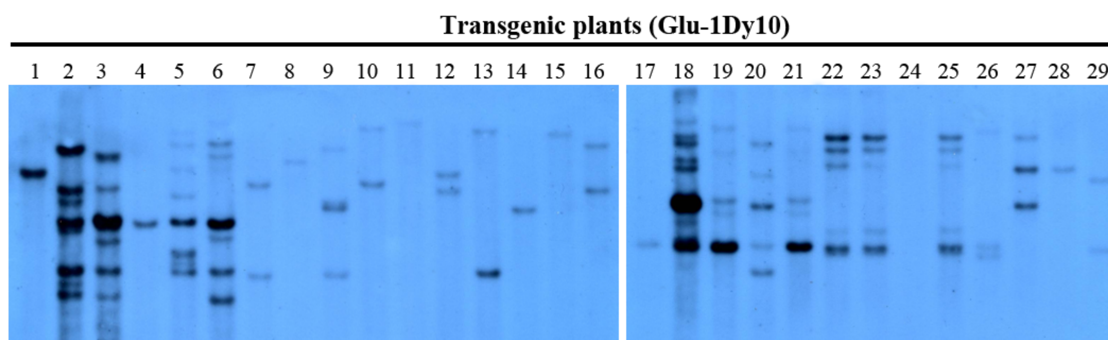

Fig. 3. Southern hybridization analysis of *Glu-1Dy10* gene from T_0 plants. The 1.35 kb fragment of *HMW* promoter was amplified by PCR using specific primer sets as the probe.

formation using two different expression cassettes was need more time consuming and effort, this method could be efficiently produce marker-free transgenic rice plants.

We performed Southern blot analysis to validate integration of *Glu-1Dy10* genes and guess segregation ratio of the marker-free plant in T_1 plants. One or multi signal bands were detected in 29 selected T_0 plants lines (Fig. 3). These results indicating that 29 selected transgenic lines were independent transgenic rice plants.

Transcript and protein analysis of *Glu-1Dy10* gene in the co-transformed rice plants

We used HMW glutenin promoter to express *Glu-1Dy10* because *Glu-1Dy10* expression in rice endosperm is important for rice flour quality. Total RNAs from randomly selected two-copy inserted T_1 transgenic seeds (13, 16, 29 lines) were extracted, and *Glu-1Dy10* gene transcript level was examined by semi-quantitative RT-PCR. The *Glu-1Dy10* transcripts were successively expressed in the T_1 generation transgenic seeds, whereas *Glu-1Dy10* expression in 'Dongjin'

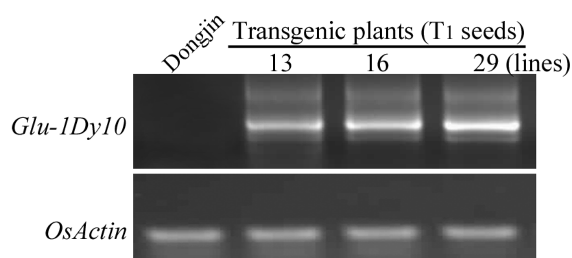

Fig. 4. Transcript analysis of the *Glu-1Dy10* gene from T_1 seeds. RT-PCR was performed with *Glu-1Dy10* T_1 seed transcripts to measure *Glu-1Dy10* mRNA expression. *OsActin* was used as a control. The reaction products of the sample plant were analyzed by electrophoresis in a 1.0% agarose gel.

was not detected (Fig. 4). *OsActin* expression was used as a quantitative control. And we analyzed the protein expression of Glu-1Dy10 by Western blot with an anti x-type HMW specific antibody. The 11 transgenic plants (2, 6, 7, 9, 15, 18, 20, 22, 24, 26, 28) which were shown abnormal morphologies comparing with 'Dongjin' were removed. After total proteins were extracted from wheat ('Jokyeong' cultivar), 'Dongjin' and transgenic plants, 0.5 μ g of wheat and 40 μ g of total protein extract of transgenic plants were used for SDS-PAGE. The immunospecificity of the anti-x-type HMW specific antibody was verified by in vivo experiment. Although the used antibody was x-type HMW specific, protein bands of Glu-1Dy10 were well detected in transgenic plants. However, the level of protein expression was not depended on their inserted copy number (Fig. 5). No protein bands were detected in one copy-inserted lines and muti-copies inserted lines. We guess that the expression levels of Glu-1Dy10 were too low to detect signal in case of the one-copy inserted lines. In case of Multi-copies inserted lines, this phenomenon may be related to homology-dependent gene silencing in plants [2]. Genetic engineering of plants sometimes results in transgene silencing after integration into the genome, which may relate to a defense mechanism against foreign DNA expression [15, 31]. Homology-dependent gene silencing has attracted considerable interest because it may be detrimental to genetic engineering and also because of its usefulness as a tool to study the mechanisms involved in detecting and inactivating exogenous DNA [15, 16].

Selection of marker-free plants harboring *Glu-1Dy10* gene in the T_1 generation

To select *Glu-1Dy10* marker-free plants harboring only the *Glu-1Dy10* gene, 72 T_1 generation seeds of the transgenic

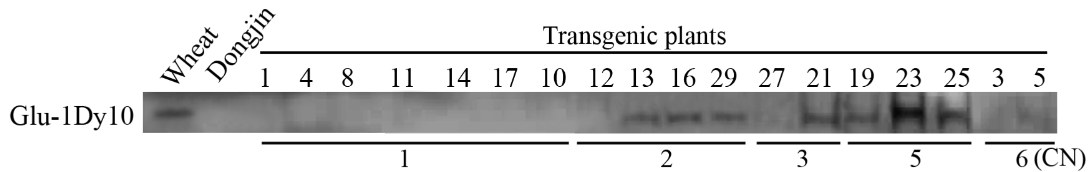

Fig. 5. Protein expression analysis of *Glu-1Dy10* gene from T_1 seeds. Western blotting was performed with an anti x-type HMW specific antibody. Total protein extracts of 0.5 μg of wheat and 40 μg of transgenic plants and 'Dongjin' were used for SDS-PAGE. CN, copy number of the *Glu-1Dy10* integrated in transgenic plants.

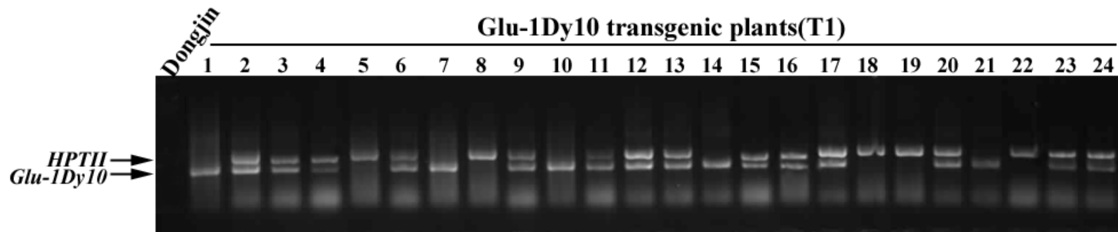

Fig. 6. PCR analysis of T_1 progenies to select marker-free transgenic plant containing *Glu-1Dy10* gene. Dongjin, non-transgenic plant as negative control; 1-24, T_1 progeny lines from T_0 plants containing both *Glu-1Dy10* and *HPTII* genes. The reaction products of the sample plant were analyzed by electrophoresis in a 1.0% agarose gel.

plant 13 were planted in soil and genomic DNA was extracted from leaves of plantlets after 4 weeks. Insertion of the *Glu-1Dy10* and *HPTII* genes was investigated by PCR analysis with *Glu-1Dy10* and *HPTII* specific primers, respectively. As shown in Fig. 6, most of the transgenic lines harbored both the *Glu-1Dy10* and *HPTII* genes, and some inserted only the *HPTII* gene. However, transgenic 1, 7, 10, 14 and 21 lines contained only the *Glu-1Dy10* gene (Fig. 6). This result shows that marker-free plants containing only the *Glu-1Dy10* gene were successfully screened at the T_1 generation. Finally, we produced marker-free transgenic rice plants harboring *Glu-1Dy10* gene. This marker-free transgenic plant harboring *Glu-1Dy10* will become useful material to optimize transgenic rice plants, which has advanced quality processing of bread and noodle by crossing with genetically engineered rice plants with other gluten genes.

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초록 : 빵과 면의 가공적성 증진을 위한 밀 저장단백질 Glu-1Dy10을 발현하는 마커프리 형질전환 벼 개발

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쌀가루는 많은 식품 가공에 이용된다. 그러나 밀가루 반죽이 빵과 면을 포함한 많은 식품 가공 제품에 적합한 반면에, 쌀로 만든 반죽은 신장성과 탄력성이 부족하다. 고분자 글루테닌 서브유닛(HMW-GS)은 밀의 가공 적성을 결정하는데 중요한 역할을 한다. 본 연구에서, 우리는 아그로박테리움(*Agrobacterium*) 동시 형질전환법을 이용하여 한국 밀 품종인 '조경'으로부터 HMW-GS를 암호화하는 밀 *Glu-1Dy10* 유전자를 발현하는 marker-free 형질전환 벼 식물체를 개발하였다. 오직 *Glu-1Dy10* 유전자와 *HPTII* (hygromycin phosphotransferase II) 저항성 유전자만을 포함하는 분리된 DNA 조각들로 구성된 두 가지 발현 카셋트(cassettes)를 독립적으로 아그로박테리움(*Agrobacterium*) EHA105 에 도입하였다. *Glu-1Dy10* 또는 *HPTII*를 함유하는 EHA105 를 각각 3:1 비율로 벼 캘러스에 접종하였다. 290개의 하이그로마이신(hygromycin) 저항성 T_0 식물체 중에서 우리는 벼 게놈에 *Glu-1Dy10*과 *HPTII* 유전자가 모두 삽입된 29개의 형질전환 라인을 획득하였다. 우리는 *Glu-1Dy10* 유전자가 벼 게놈 내로 도입된 것을 Southern blot 분석을 통해 다시 확인하였다. 형질전환 벼 종자에서 *Glu-1Dy10*의 전사(Transcripts)와 단백질을 semi-quantitative RT-PCR과 Western blot 분석을 통해서 확인하였다. 최종적으로, 오직 *Glu-1Dy10* 유전자를 갖는 marker-free 식물체를 T_1 세대에서 성공적으로 선발할 수 있었다.