ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science **2013** Vol. 23. No. 11. 1317~1324 DOI : http://dx.doi.org/10.5352/JLS.2013.23.11.1317

Development of Marker-free Transgenic Rice for Increasing Bread-making Quality using Wheat High Molecular Weight Glutenin Subunits (HMW-GS) Gene

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Received October 10, 2013 /Revised November 8, 2013 /Accepted November 11, 2013

High-molecular weight glutenin subunits (HMW-GS) have been shown to play a crucial role in determining the processing properties of the wheat grain. We have produced marker-free transgenic rice plants containing a wheat *Glu-1Bx7* gene encoding the HMG-GS from the Korean wheat cultivar 'Jokyeong' using the *Agrobacterium*-mediated co-transformation method. The *Glu-1Bx7*-own promoter was inserted into a binary vector for seed-specific expression of the *Glu-1Bx7* gene. Two expression cassettes comprised of separate DNA fragments containing only *Glu-1Bx7* and hygromycin phosphotransferase II (*HPTII*) resistance genes were introduced separately to the *Agrobacterium tumefaciens* EHA105 strain for co-infection. Each EHA105 strain harboring *Glu-1Bx7* or *HPTII* was infected to rice calli at a 3:1 ratio of *Glu-1Bx7* and *HPTII*, respectively. Then, among 216 hygromycin-resistant T_0 plants, we obtained 24 transgenic lines with both *Glu-1Bx7* and *HPTII* genes inserted into the rice genome. We reconfirmed integration of the *Glu-1Bx7* gene into the rice genome by Southern blot analysis. Transcripts and proteins of the wheat *Glu-1Bx7* were stably expressed in the rice T_1 seeds. Finally, the marker-free plants harboring only the *Glu-1Bx7* gene were successfully screened at the T_1 generation.

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

Introduction

Rice quality has become an important consideration due to the growing demands of local and world food markets. The increasing population of rice consumers, especially in Africa, Asia, and Southeast Asia, depend solely on this cereal food. Rice flour is used in many food products and improving rice quality is of great relevance to many Asian countries. However, dough made from rice lacks extensibility and elasticity, whereas that of wheat is suitable for many food products including breads.

Wheat flour is different from other cereal flours, including rice, because it contains gluten that gives it the elasticity and extensity required for bread-making [5]. Gluten consists mainly of two types of seed storage proteins, the glutenins and the gliadins. Glutenins are classified into high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS). Although the HMW-GS contribute only about 5% of the total protein in mature wheat kernels [26], the elasticity of wheat dough depends mainly on the HMW-GS, so their networking is important determinants of bread-making quality [19, 20].

The HMW-GS are encoded by the Glu-A1, Glu-B1 and glu-D1 genes on the long arm of chromosomes 1A, 1B and 1D, respectively [21]. Each locus includes two genes linked together encoding two different types of HMW-GS, x- and y-type subunits [20, 22]. 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].

Development of transgenic plants increasing bread-making quality using various HMW-GS genes is good way to solve lacks of rice extensibility and elasticity. Araki's [4] group has developed transgenic rice using transformation-competent artificial chromosome (TAC) clones harboring a HMW-GS and LMW-GS genes from wheat genomic DNA. All transgenic lines had HMW-GS subunit proteins in the rice endosperm, and the expressed proteins are processed at the same site as the mature protein in wheat seeds.

The persistence of selectable marker genes in transgenic crops destined for field cultivation and human food leads to serious public concerns about the safety of transgenic

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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. 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. 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.

In this study, we generated marker-free transgenic rice expressing the wheat HMW-GS protein without any herbicide or antibiotic resistance marker genes using the co-transformation method. The marker-free transgenic plant expressing *Glu-1Bx7* 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-1Bx7* glutenin gene

'Jokyeong' (*Triticum aestivum* L. cv. Jokyeong) was used for cloning the *Glu-1Bx7* gluenin gene. The *Glu-1Bx7* gene was amplified by polymerase chain reaction (PCR) of genomic DNA using the primers *Bx7-CF* (primer sequences: 5'-AGGGTACCGAGATGGCTAAGCGCCTGG-3') and *Bx7-CR* (primer sequences: 5'-GATCTAGATCACTGCCTGGT-CGACAATG-3'), which were designed from a sequence on GenBank (accession no. X13927). 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 first inserted *Glu-Bx7*-own promoter from wheat cultivar 'Jokyeong' 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 *Xho*I and *EcoR*I restriction enzyme treatment. After klenow enzyme treatment for blunt ligation, the vector was self-ligated. Then, amplified the *Glu-1Bx7* gene with the *EcoR*I and *Kpn*I restriction enzyme sites was constructed into *pBTEX* binary vectors under the control of *Glu-1Bx7*-own promoter (Fig. 1A). The positive selectable marker cassette for co-transformation was used by an empty *pBTEX* binary vector (Fig. 1B).

Agrobacterium handling

Competent Agrobacterium tumefaciens EHA105 was transformed with *Glu-1Bx7*- 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 pre-incubated 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-1Bx7* 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-1Bx7*; forward 5′- AGGGTACCGA-GATGGCTAAGCGCCTGG -3′, reverse 5′- GATCTAGAT-CACTGCCTGGTCGACAATG -3′, *HPTII*; forward 5′- CGCT-TCTGCGGGCGATTT -3′, reverse 5′- CCCATTCGGACCG-CAAGGA -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 [29]. 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 [32]. 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-1Bx7* forward 5′- AGGGTACCGAGATGGCTAAGCG-CCTGG -3′, *Glu-1Bx7* reverse 5′ GATCTAGATCACTGC-CTGGTCGACAATG -3′; *OsActin* primers were used as internal standards for mRNA expression profiling [17, 31]. 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′- GGA ACT GGT ATG GTC AAG GC -3′ and 5′-AGT CTC ATG GAT ACC CGC AG -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 50 mM 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

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

To improve improved dough properties of rice flour, we cloned high-molecule weight glutenin subunit gene (*Glu-1Bx7*) from genomic DNA of *Triticum aestivum* cv. Jokyeong by PCR analysis with specific primers. We tried to generate marker-free transgenic plant expressing only *Glu-1Bx7* gene on rice through *Agrobacterium*-mediated co- transformation system. To make a marker-free vector, we first remove the *HPTII* expression cassette (*CaMV 35S* promoter-*HPTII* gene-*CaMV 35S* terminator) by treatment of *XhoI* and *EcoRI* restriction enzymes and inserted wheat *Glu-1Bx7*-own promoter. And then *Glu-1Bx7* gene was constructed under the control of *Glu-1Bx7*-own promoter into *pBTEX* vector, which was modified *pCAMBIA1300* binary vector (Fig. 1A). And original *pCAMBIA1300* binary vector harboring *HPTII* gene was used to select hygromycin resistant T_0 plants (Fig. 1B). 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-1Bx7* and the *HPTII*, and the *HPTII* and *Glu-1Bx7* genes were validated by PCR analysis.

Generation of marker-free *Glu-1Bx7* transgenic rice plants

Each EHA105 strain harboring *Glu-1Bx7* expression vector or *HPTII* expression vector was cultured in YEP medium for plant transformation. The cultured cells were re-suspended to OD_{600}=0.1 in AAM medium [11], and each *Glu-1Bx7* and *HPTII* cell was added at a 3:1 ratio. These

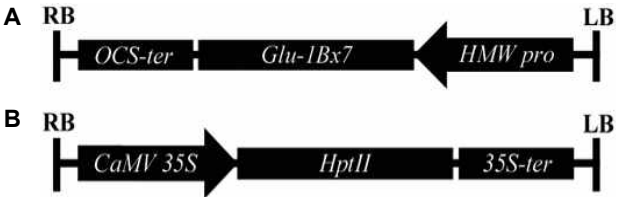

Fig. 1. Vector constructs expressing the *Glu-1Bx7* (upper panel) and hygromycin phosphotransferase II (*HPTII*) (lower panel) genes in the binary vectors. HMW pro, *Glu-1Bx7*-own promoter; OCS-ter, octopine synthase terminator; CaMV 35S, cauliflower mosaic virus promoter; 35S-ter, 35S terminator; RB, right border; LB, left border.

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

Gene	No. of T_0 plants	No. of plants containing the *Glu-1Bx7*	Frequency of co-transformation (%)
Glu-1Bx7	216	24	11.1

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 216 independent hygromycin-resistant T_0 plants through co-infection in the *Agrobacterium* transformation system.

Genomic DNA from 216 independent T_0 plants was extracted and insertion of *HPTII* and *Glu-1Bx7* genes was analyzed using PCR analysis with gene specific primers. As shown in Fig. 2, *HPTII* gene in all of T_0 plants was amplified, but no PCR products of 'Jokyeong' and 'Dongjin' used as negative controls were detected. Next, we investigate the insertion of *Glu-1Bx7* gene into rice genome within T_0 plants by PCR analysis. Among 216 independent transgenic lines, *Glu-1Bx7* gene in 24 T_0 plants was amplified PCR product which is same PCR product size of 'Jokyeong'and plasmind used as positive controls (Fig. 2). This result means that 24 transgenic lines harbored both *Glu-1Bx7* and *HPTII* genes. And the frequency co-transformation was about 11.1% in our experimental system (Table 1). We performed Southern blot analysis with the *Glu-1Bx7*-own promoter as probes to validate their insertion and guess segregation ratio of the marker-free plant in T_1 plants. One or seven signal bands in 24 selected T_0 plants lines were detected (Fig. 3).

Transcript and protein analysis of *Glu-1Bx7* gene in the co-transformed rice plants

Because *Glu-1Bx7* expression in rice endosperm is important for rice flour quality and we used *Glu-1Bx7*-own promoter to express *Glu-1Bx7*, total RNA from one copy-inserted T_1 generation transgenic seeds was extracted, and *Glu-1Bx7* gene transcript level was examined by semi-quantitative RT-PCR. The *Glu-1Bx7* transcripts were successively expressed in the T_1 generation transgenic seeds, whereas *Glu-1Bx7* expression in 'Dongjin' was not detected (Fig. 4). *OsActin* expression was used as a quantitative control. And we analyzed the protein expression of Glu-1Bx7 by Western blot with an anti x-type HMW specific antibody. The six transgenic plants (4, 17, 18, 19, 20 and 22) which were shown abnormal morphologies comparing with 'Dongjin' were removed. After total protein extraction from wheat ('Jokyeong' cultivar), 'Dongjin' and transgenic plants, 0.5 μg of wheat and 40 μg 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 protein bands were well detected in transgenic plants, however, the level of protein expression was not depended on their inserted copy number (Fig. 5). Multi- copies of *Glu-1Bx7* gene were inserted into genome in some transgenic plants (5, 23 and 16), no proteins were detected. We guess that this phenomenon is homol-

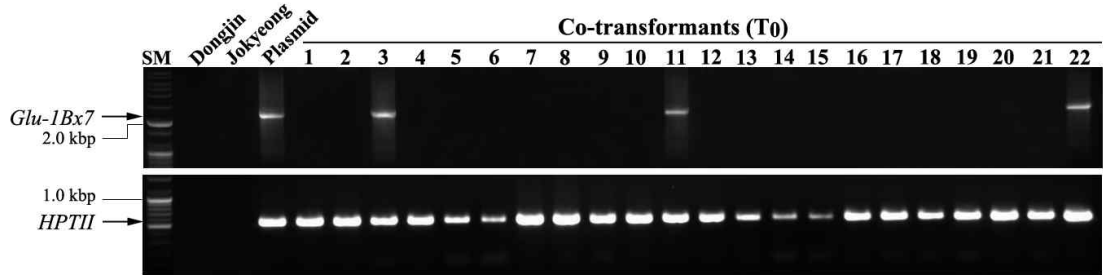

Fig. 2. Identification of T_0 plants by gene specific primer sets. *Glu-1Bx7* (upper panel) and *HPTII* (lower panel) genes were amplified using *Glu-1Bx7* and *HPTII* specific primer sets, respectively. SM, molecular marker; 'Dongjin' (Korean rice cultivar), non-transgenic plant; Jokyeong, Korean wheat cultivar; Plasmid, vector construct containing *Glu-1Bx7* and *HPTII* genes; 1-22, co-transformant transgenic lines. Genomic DNAs from each plant were used as the template for *Glu-1Bx7* and *HPTII* specific amplification. The reaction products of the sample plant were analyzed by electrophoresis on a 1.0% agarose gel.

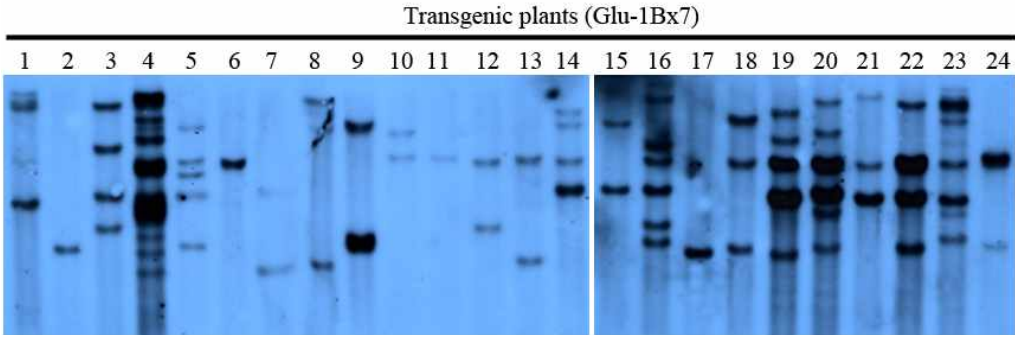

Fig. 3. Southern hybridization analysis of *Glu-1Bx7* gene from T_0 plants. The 1.35 kb fragment of *Glu-1Bx7*-own promoter was amplified by PCR using specific primer sets as the probe.

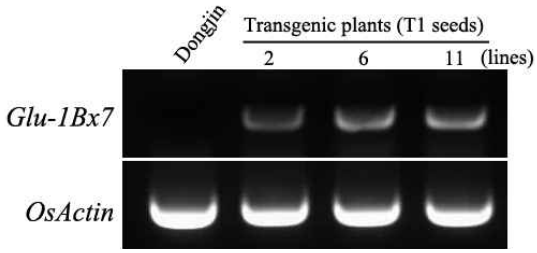

Fig. 4. Transcript analysis of the *Glu-1Bx7* gene from T_1 seeds. RT-PCR was performed with *Glu-1Bx7* T_1 seed transcripts to measure *Glu-1Bx7* mRNA expression. Gene-specific PCR primers (forward and reverse primers) were designed to amplify the *Glu-1Bx7* gene. *OsActin* was used as a control. The reaction products of the sample plant were analyzed by electrophoresis in a 1.0% agarose gel.

ogy-dependent gene silencing in plants [2].

Selection of marker-free plants harboring *Glu-1Bx7* gene in the T_1 generation

To select *Glu-1Bx7* marker-free plants harboring only the *Glu-1Bx7* gene, 90 T_1 generation seeds of the transgenic plant 8 were planted in soil and genomic DNA was extracted from leaves of plantlets after 4 weeks. Insertion of the *Glu-1Bx7* and *HPTII* genes was investigated by PCR analysis. As shown in Fig. 6, most of the transgenic lines harbored both the *Glu-1Bx7* and *HPTII* genes, and some inserted only the *HPTII* gene. However, transgenic 3, 10, 18 and 21 lines contained only the *Glu-1Bx7* gene (Fig. 6). This result shows that marker-free plants containing only the *Glu-1Bx7* gene were successfully screened at the T_1 generation. Finally, we produced marker-free transgenic rice plants harboring *Glu-1Bx7* gene for advanced quality processing of bread and noodle.

Discussion

Wheat gluten proteins are classified into two broad groups based on their aggregation and functional properties, including the glutenins, which form polymers stabilized by inter-chain disulfide bonds, and gliadins, which are present as monomers and interact by non-covalent forces [27]. In particular, HMW-GSs are minor components in terms of quantity, but they are key factors during bread making because they are major determinants of gluten elasticity [28] by promoting the formation of larger glutenin polymers.

In this study, we cloned *Glu-1Bx7*, which is one of HMW-GS genes, and validated the insertion of the *Glu-1Bx7* gene in T_0 plants through PCR analysis with gene specific primers and Southern blot analysis (Fig. 2, 3). The *Glu-1Bx7*-own promoter was introduced for seed specific expression of *Glu-1Bx7* gene. The transcript and protein of

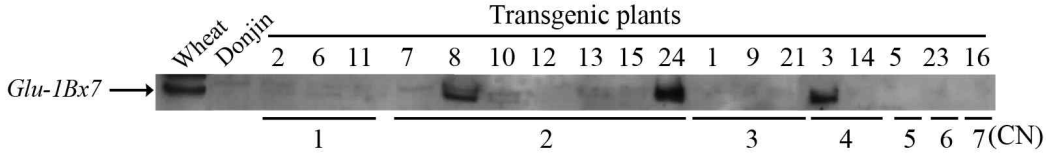

Fig. 5. Protein expression analysis of *Glu-1Bx7* gene from T_1 seeds. Western blotting was performed with an anti x-type HMW specific antibody. Total protein extracts of 0.5 μg of wheat and 40 μg of transgenic plants and 'Dongjin' were used for SDS-PAGE. CN, copy number.

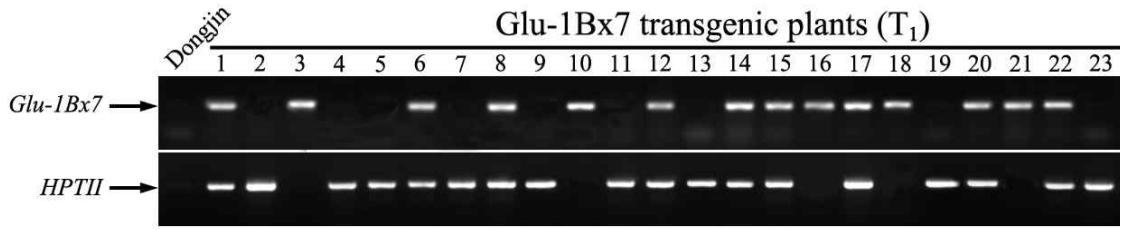

Fig. 6. PCR analysis of T_1 progenies to select marker-free transgenic plant containing *Glu-1Bx7* gene. Dongjin, non-transgenic plant as negative control; 1-23, T_1 progeny lines from T_0 plants containing both *Glu-1Bx7* and *HPTII* genes. The reaction products of the sample plant were analyzed by electrophoresis in a 1.0% agarose gel.

Glu-1Bx7 in the transgenic plants were stably expressed in T_1 generation rice seeds (Fig. 4). This result suggested that the protein processing system was conserved between rice and wheat. However, the level of protein expression was not depended on their inserted copy number (Fig. 5). 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, 30]. This phenomenon may be related to homology-dependent gene silencing in plants. 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].

Multi- copies of *Glu-1Bx7* gene were inserted into genome in some transgenic plants (5, 23 and 16), no proteins were detected. We guess that this phenomenon is homology-dependent gene silencing in plants [2].

The co-transformation frequency in our experimental conditions was 11.1% (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-transformation using two different expression cassettes was need more time consuming and effort, this method could be efficiently produce marker-free transgenic rice plants.

Finally, we obtained marker-free transgenic plants containing only *Glu-1Bx7* gene from each of the T_1 plants (Fig. 6). This marker-free transgenic plant harboring *Glu-1Bx7* 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.

Acknowledgments

This work was supported by a grant from the Department of Functional Crop, NICS, RDA (No. PJ008717), Republic of Korea. We would like to thanks sincerely to Dr. Paul Scott's assistance of USDA-ARS for offering anti-x-type HMW specific antibody.

References

1. Altpeter, F., Vasil, V., Srivastava, V. and Vasil, I. K. 1996. Integration and expression of the high-molecular-weight glutenin subunit 1Ax1 gene into wheat. *Nat Biotechnol* **14**, 1155-1159.
2. Alvarez, M. L., Guelman, S., Halford, N. G., Lusting, S., Reggiardo, M. I., Ryabushkina, N., Shewry, P., Stein, J. and Vallejos, R. H. 2000. Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. *Theor Appl Genet* **100**, 319-327.
3. An, G., Evert, P. R., Mitra, A. and Ha, S. B. 1988. Binary vectors. pp. A3/1-19, In: Gelvin, S. B. and. Schilperoort, R. A. (eds.), *Plant molecular biology manual.* Kluwer Academic Publishers, Boston, MA.
4. Araki, E., Ikeda, M. T., Ohgihara, Y., Toyoda, A. and Yano, H. 2008. Development of transgenic rice (*Oryza sativa* L.) expressing wheat high- and low-molecular-weight glutenin subunit proteins. *Breed Sci* **58**, 121-128.
5. Barro, F., Rooke, L., Bekes, F., Gras, P., Tatham, A. S., Fido, R., Lazzeri, P. A., Shewry, P. R. and Barcelo, P. 1997. Transformation of wheat with high-molecular-weight subunit genes results in improved functional properties. *Nat Biotechnol* **15**, 1295-1299.
6. Blechl, A. E. and Anderson, O. D. 1996. Expression of a novel highmolecular-weight glutenin gene in transgenic wheat. *Nat Biotechnol* **14**, 875-879.
7. Chen, H., Nelson, R. S. and Sherwood, J. L. 1994. Enhanced recovery of transformants of Agrobacterium tumefaciens after freeze-thaw transformation and drug selection. *Biotechniques* **16**, 664-669.
8. Cho, J. I., Ryoo, N., Ko, S., Lee, S. K., Lee, J., Jung, K. H., Lee, Y. H., Bhoo, S. H., Winderickx, J., An, G., Hahn, T. R. and Jeon, J. S. 2006. Structure, expression, and functional

analysis of the hexokinase gene family in rice (*Oryza sativa* L.). *Planta* **224**, 598-611.
9. Chu, C. C., Wang, C. S., Sun, C. S., Hsu, C., Yin, K. C., Chu, C. Y. and Yun, B. F. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci China Math* **18**, 659-668.
10. Galili, G. 1989. Heterologous expression of a wheat high-molecular-weight glutenin gene in *Escherichia coli*. *Proc Natl Acad Sci USA* **86**, 7756-7760.
11. Hiei, Y., Ohta, S. Komari, T. and Kumashiro, T. 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.
12. Hohn, B., Levy, A. A. and Puchta, H. 2001. Elimination of selection markers from transgenic plants. *Curr Opin Biotechnol* **12**, 139-143.
13. Komari, T., Hiei, Y., Saito, Y., Murai, N. and Kumashiro, T. 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by Agrobacterium tumefaciens and segregation of transformants free from selection markers. *Plant J* **10**, 165-174.
14. Kuiper, H. A., Kleter, G. A., Noteborn, H. P. and Kok, E. J. 2001. Assessment of the food safety issues related to genetically modified foods. *Plant J* **27**, 503-528.
15. Kumpatla, S. P., Chandrasekharan, M. B., Iyer, L. M., Li, G. and Hall, T. C. 1998. Genome intruder scanning and modulation systems and transgene silencing. *Trends Plant Sci* **3**, 97-104.
16. Matzke, M. A., Matzke, J. M. and Eggleston, W. B. 1996. Paramutation and transgene silencing: a common response to invasive DNA? *Trends Plant Sci* **1**, 382-388.
17. McElroy, D., Zhang, W., Cao, J. and Wu, R. 1990. Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* **2**, 163-171.
18. Park, S. K., Jung, Y. J., Lee, J. R., Lee, Y. M., Jang, H. H., Lee, S. S., Park, J. H., Kim, S. Y., Moon, J. C., Lee, S. Y., Chae, H. B., Shin, M. R., Jung, J. H., Kim, M. K., Kim, Y. Y., Yun, D. J., Lee, G. O. and Lee, S. Y. 2009. Heat-shock and redox-dependent functional switching of an h-type arabidopsis thioredoxin from a disulfide reductase to a molecular chaperone. *Plant Physiol* **150**, 552-561.
19. Payne, P. I., Corfield, K. G. and Blackman, J. A. 1979. Identification of a high-molecular-weight subunit of glutenin whose presence correlates with breadmaking quality in wheats of related pedigree. *Theor Appl Genet* **55**, 153-159.
20. Payne, P. I., Corfield, K. G., Holt, L. M. and Blackman, J. A. 1981. Correlations between the inheritance of certain high-molecular-weight subunits of glutenin and breadmaking quality in progenies of six crosses of bread wheat. *J Sci Food Agric* **32**, 51-60.
21. Payne, P. I., Law, C. N. and Mudd, E. E. 1980. Control by homologous group 1 chromosomes of the high-molecular-weight subunits of glutenin, a major protein of wheat endosperm. *Theor Appl Genet* **58**, 113-120.
22. Payne, P. I., Nightingale, M. A., Krattiger, A. F. and Holt, L. M. 1987. The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. *J Sci Food Agric* **40**, 51-65.
23. Roberts, L. S., Thompson, R. D. and Flavell, R. B. 1989. Tissue-specific expression of a wheat high-molecular-weight glutenin gene in transgenic tobacco. *Plant Cell* **1**, 569-578.
24. Rooke, L., Barro, F., Tatham, A. S., Fido, R., Steele, S., Bekes, F., Gras, P., Martin, A., Lazzeri, P. A., Shewry, P. R. and Barcelo, P. 1999. Altered functional properties of tritordeum by transformation with HMW glutenin subunit genes. *Theor Appl Genet* **99**, 851-858.
25. Ramessar, K., Peremarti, A., Go´mez-Galera, S., Naqvi, S., Moralejo, M., Muñoz, P., Capell, T. and Christou, P. 2007. Biosafety and risk assessment framework for selectable marker genes in transgenic crop plants: a case of the science not supporting the politics. *Transgenic Res* **16**, 261-280.
26. Shewry, P. R., Halford, N. G. and Tatham, A. S. 1989. The high-molecularweight subunits of wheat, barley and rye: genetics, molecular biology, chemistry and role in wheat gluten structure and functionality. *Oxford Surveys Plant Mol Cell Biol* **16**, 163-219.
27. Shewry, P. R., Tatham, A. S., Fido, R., Jones, H., Barcelo, P. and Lazzeri, P. A. 2001. Improving the end use properties of wheat by manipulating the grain protein composition. *Euphytica* **119**, 45-48.
28. Tatham, A. S. and Shewry, P. R. 1985. The conformation of wheat gluten proteins. The secondary structures and thermal stabilities of alpha-, beta-, gamma- and omega- gliadins. *J Cereal Sci* **3**, 103-113.
29. Thompson, B. G., Anderson, R. and Murray, R. G. 1980. Unusual polar lipids of Micrococcus radiodurans strain Sark. *Can J Microbiol* **26**, 1408-1411.
30. Vaucheret, H., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., Mourrain, P., Palauqui, J. C. and Vernhettes, S. 1998. Transgene-induced gene silencing in plants. *Plant J* **16**, 651-659.
31. Volkov, R. A., Panchuk, I. I. and SchoZ, F. 2003. Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. *J Exp Bot* **54**, 2343-2349.
32. Zhiwu, Li. and Harold, N. T. 2005. Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch. *Biotechniques* **38**, 872-876.

초록 : 밀 고분자 글루테닌 유전자를 이용하여 빵 가공적성 증진을 위한 마커 프리 형질전환 벼의 개발

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고분자 글루테닌 서버유닛(high molecular-weight glutenin subunit, HMW-GS)은 밀의 가공적성을 결정하는데 중요한 역할을 수행한다. 우리는 *Agrobacterium* 동시 형질전환법을 이용하여 한국 밀 품종인 '조경'으로부터 밀 HMW-GS을 암호화하는 *Glu-1Bx7* 유전자를 가지는 marker-free 형질전환 벼를 생산하였다. *Glu-1Bx7* 유전자의 종자 특이적 발현을 위하여 밀 *Glu-1Bx7* 유전자 자체 프로모터를 벡터 내에 삽입하였다. 동시 접종을 위해서 오직 *Glu-1Bx7* 유전자와 hygromycin phosphotransferase II (*HPTII*) 저항성 유전자만으로 구성된 두 종류의 발현 카셋트를 독립적으로 *Agrobacterium* EHA105에 도입하였고, *Glu-1Bx7*와 *HPTII*가 도입된 각각의 EHA105 *Agrobacterium*을 3:1 비율로 혼합하여 벼 캘러스에 접종하였다. 216개의 *HPTII* 저항성 형질전환체 중에서 벼 게놈에 *Glu-1Bx7*과 *HPTII*가 모두 삽입된 24개의 형질전환 라인을 획득하였다. *Glu-1Bx7*와 *HPTII*가 벼 게놈에 도입된 것을 Southern blot을 통해서 다시 확인하였다. 형질전환 벼 T_1 세대의 종자에서 밀 *Glu-1Bx7* 유전자가 전사와 번역되어 오직 *Glu-1Bx7*만을 가지는 marker-free 식물체를 T_1 세대에서 성공적으로 선발할 수 있었다.