Genetic Transformation of a High Molecular Weight Glutenin (Glu-1Dx5) to Rice cv. Fatmawati

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Abstract: In order to improve rice dough functionality, we co-transformed the Glu-1Dx5 gene encoding a high molecular weight (HMW) glutenin subunit Dx5 from bread wheat, Triticum aestivum L. and either bar gene conferring resistance to herbicide bialaphos or hpt gene conferring resistance to hygromycin B to rice callus cells of cv. Fatmawati. We molecularly characterized 9 plants regenerated from bialaphos-containing medium and 63 plants from hygromycin-containing medium. The Glu-1Dx5 gene was detected by PCR analysis in 15 transgenic T0 plants. Further analysis of T1 and T2 plants revealed that some transgenic plants carried the Glu-1Dx5 gene. Analysis of the endosperm extracts of T2 plants by SDS-PAGE revealed the existence of a protein similar in size to the wheat Glu-1Dx5 gene product, suggesting successful expression of the transgene. These plants will be incorporated into breeding program for further assessment of their benefits.

Key words: Genetic transformation, Glu-1Dx5, High molecular weight glutenin, Rice.
genetic transformation method. Genetic transformation of rice using particle bombardment has offered some attractive objectives such as to obtain a large number of transformed plants (Dai et al., 2001), to get high transgene copy number (Dai et al., 2001; Shou et al., 2004), to introduce simultaneous multiple genes which are valuable for designing the metabolic pathway (Maqbool and Christou, 1999; Agrawal et al., 2005), plastid or chloroplast transformation (Daniel et al., 2001; Kanamoto et al., 2006), and to introduce the gene cassette without the sequence of vector (Breitler et al., 2001; Kanamoto et al., 2006), and to introduce the gene cassette without the sequence of vector (Breitler et al., 2001). Other advantages of particle bombardment are the lack of biological constraints and host limitations, efficient targeting of diverse cell types and delivery of HMW DNA (Altpeter et al., 2005). We have revealed the most favorable conditions for using the HeliosTM gene gun device on rice callus (Carsono and Yoshida, 2008). The object of this experiment was to introduce the Glu-1Dx5 gene into rice cv. Fatmawati. This gene either with the bar gene conferring resistance to herbicide bialaphos or with the hpt gene encoding resistance to hygromycin B as a selectable marker has been co-transformed to rice callus cells of cv. Fatmawati using the Helios gene gun device (BioRad Lab, USA). Glu-1Dx5 gene expression was analyzed in rice endosperm extracts by SDS-PAGE using transgenic T2 plants.

Materials and Methods

1. Preparation of target tissues
Callus induction and maintenance procedures were as reported previously (Carsono and Yoshida, 2006) and callus derived from scutellar tissues of mature seed of cv. Fatmawati was used for genetic transformation experiments because it has high regeneration capacity (Carsono and Yoshida, 2006), and does not induce somaclonal variants in the spikelet-related traits (Carsono and Yoshida, 2007).

2. Plasmid DNA used for bombardment
Plasmid pK+Dx5B (Anderson et al., 2002) and either pAHC25 (Christensen et al., 1996) or pIG001 were used for bombardment. The pK+Dx5B provided by Dr. Ann Blechl (USDA, US) contains an 8.2 kb genomic DNA fragment from hexaploid bread wheat (cv. Cheyenne) that includes the Glu-1Dx5 coding sequence (GenBank Accession Number X12928; Fig. 1), 3.2 kb of 5’ flanking sequence and 1.2 kb of 3’ flanking sequence (Halford et al., 1992). Plasmid pAHC25 contains bar gene (Thompson et al., 1987) from Streptococcus hygroscopicus conferring resistance to herbicide bialaphos/phosphinothricin acetyl transferase (PAT), while pIG001 contains hpt gene conferring resistance to hygromycin B. Previously UidA gene in pAHC25 has been removed by cleaving with BamHI then self-ligated, and resulting plasmid was named pBar25. The Glu-1Dx5 gene was driven by its own promoter, while promoter of maize ubiquitin-1 (Christensen and Quail, 1996) and the 35S promoter of cauliflower mosaic virus were used to drive the bar gene and hpt gene, respectively. Plasmid was amplified in E. coli DH5α cells and purified by the alkaline-SDS lysis method (Sambrook et al., 1989), followed by PEG/NaCl treatment. The plasmids used for bombardment were confirmed by restriction digestion analysis and checked by agarose gel electrophoresis. The concentration of plasmid-DNA was measured with a spectrophotometer (Gene Spec III, Naka Instruments Co., Ltd., Japan).

3. Helios gene gun bombardment procedures
The bombardment procedure for Helios gene gun was as reported previously (Carsono and Yoshida, 2008). The parameters used in this study were 250 psi Helium pressure (equal to 1,724 Kpa), 0.6 μm gold particle diameter, 0.25 μg gold particles per shot, and 0.4 M mannitol of osmotic medium. The amount of plasmid-DNA per shot was 1.0 μg for pK+Dx5B and pBar25, while 1.5 μg for pK+Dx5B and pIG001. The molar ratio used was 1:1 for both co-transformation events. Eight embryogenic calluses were placed on filter paper in the center of a Petri dish, and covered with a plastic bowl with a cavity the size of the spacer of the Helios gene gun. These calluses were bombarded once, then immediately transferred to osmotic medium and incubated in the dark at 26°C. Osmotic treatment of callus cells with 0.4 M mannitol on medium NB, was applied for 4–6 hr before and 16 hr after bombardment.

4. Selection and regeneration of transformed calluses
Immediately after exposure to osmotic treatment, the calluses were transferred to NB subculture medium and subcultured for one wk. The same medium except for addition of 4 mg L⁻¹ bialaphos or 50 mg L⁻¹ hygromycin was used for callus selection. Two to three successive selections were performed. Resistant calluses as shown by arrows in Fig. 2a, b, were then transferred to pre-regeneration and subsequently to regeneration media.

5. Molecular characterization of transgenic rice plants
Genomic DNA was extracted from leaves of primarily
transformed and untransformed plants using Plant DNA extraction kit (Nucleon Phyto Pure, Amersham, USA). The primer design, in silico PCR and restriction analysis were always carried out using FastPCR software (Kalendar, 2007). PCR was carried out with 20–50 ng of genomic DNA, in reaction mixture containing 5 μL KOD Dash 10xPCR buffer, 5 μL of 2mM dNTPs, 1 μL each primer (1–20 μM) and 0.5 μL of 1.25 units KOD DNA polymerase (Toyobo Co., LTD, Osaka, Japan). PCR amplification was carried out for the Glu-1Dx5 coding sequence (upper primer 5’-CTGGATCCATGCAGGCTACC-3’; lower primer 5’-AAGGATCCAGACATGCAGC-3’). The PCR conditions were as follows: 30 cycles for expected amplified product 3.2 kb in size (94ºC for 30 sec, 59ºC for 4 sec, 74ºC for 1 min. 25 s, and final extension at 74ºC for 5 min.). DNA amplification was carried out in a final volume of 50 μL. PCR products were examined by electrophoresis through a 1% agarose in 1x TBE buffer.

6. Expression of Glu-1Dx5 in transgenic rice endosperm
Endosperm materials from wild-type and transgenic T2 lines were ground to a powder in liquid nitrogen. Total storage proteins in the rice endosperm were extracted with 50mM Tris-HCl (pH 8.0) containing 2% of SDS, 50% of 1-propanol and 1% of dithiothreitol, as described by Araki et al. 2008. Proteins were precipitated in cold 80% acetone. The pellets were air dried and resuspended in SDS buffer containing 10mM Tris-HCl (pH 6.8), 1% of SDS, 1% of β-mercaptoethanol and 20% of glycerol and then heated in boiling water for 2 min. Extracted proteins (about 20 μg per each lane) were separated by electrophoresis on a 10% SDS-PAGE (Laemmli, 1979). The gel was stained with Coomassie Brilliant Blue R-250.

Results and Discussion
1. Selection of putative transgenic rice calluses
Approximately 100 calluses were bombarded for two co-transformation events (pK+Dx5B either with pBar or pIG001). Approximately 12 calluses were regenerated on bialaphos-containing medium, while 18 calluses were regenerated on hygromycin-containing medium. A major problem during the course of transformation was a necrotic response in calluses after transfer to selection medium containing 4 mg L-1 bialaphos (Fig. 2a) or 50 mg L-1 hygromycin (Fig. 2b). The growth of the bombarded callus cluster was severely inhibited and the calluses gradually turned brown. Mostly their growth became retarded after exposure to bialaphos or hygromycin for 2-3 wk in the selection medium. During the regeneration experiment, shoots were abundantly obtained, but green plantlets were obviously rare, especially on calluses that were bombarded with pK+Dx5B and pBar25 (growing on bialaphos-containing medium). Transformation efficiency may be suboptimal in the presence of toxic substrates like bialaphos or hygromycin, because dying untransformed cells may inhibit transformed cells from proliferating by secreting inhibitors or preventing transport of essential nutrients to the living transformed cells (Haldrup et al., 1998). After two to three subcultures on bialaphos-containing NB5 medium, 11 putative transgenic rice plants were regenerated, but only 9 plants were able to grow further (Fig. 3a). By contrast, approximately 63 plants were obtained from hygromycin-containing NB5 medium and they were able to grow further (Fig. 3b). According to the number of putative transgenic plants obtained, we propose that the hpt gene is better than the bar gene as a selective marker. These transgenic plants were grown in a glass house at the Genomic Center, Utsunomiya University.

2. Molecular detection of the wheat Glu-1Dx5 gene in putative transgenic plants
Molecular screening by means of PCR of T0 and T1 plants revealed that some lines contain the Glu-1Dx5 coding sequence, though some not (Fig. 4). Using primers that were designed specifically to amplify the 3.2 kb of expected DNA fragment, the Glu-1Dx5 gene was
successfully detected. However, this fragment was not detected in the untransformed plant (negative control), while this band was detected in the positive control using plasmid pK+Dx5 as template (Fig. 4). Out of 9 T₀ plants regenerated from bialaphos-containing medium, 5 plants (56%) were positive for Glu-1Dx5 gene, while 59 out of 63 plants (94%) carried this transgene. However, further analysis of 21 T₁ lines revealed only 3 lines (14%) positive. This might be due to segregation in T₁ and limited number of plants analyzed. This result shows that a 3.2 kb of 5' flanking sequence of the Glu-1Dx5 has a promoter that can drive the gene for transcription. To confirm the existence of the promoter in the full-length of 8.2 kb Glu-1Dx5, we examined the DNA sequence with TSSP-Prediction of Plant Promoter Software (Softberry Inc.), and found at least 4 promoters or enhancers on the basis of TATA box prediction, suggesting that the own promoter of Glu-1Dx5 is able to perform the transcription.

3. Expression of Glu-1Dx5 in transgenic rice endosperm

Seventeen T₂ lines were tested by PCR. Each line consisted of four to seven plants. The number of lines in which at least 2 plants had a Glu-1Dx5 product was 7. The number of T₂ lines in which all plants were positive was 4. To examine the expression of Glu-1Dx5 in these T₂ lines, we analyzed the endosperm extracts by SDS-PAGE. The T₂ endosperm contained a protein that migrated near the 105 kDa marker, which was not detected in wild-type endosperm (Fig. 5). The protein detected in our transgenic T₂ endosperm was similar in size to the wheat Glu-1Dx5 product detected in transformed maize plants (Sangtong, 2002). Araki et al. (2008) suggested that the protein-processing system was conserved in both rice and wheat, and that it was possible to produce wheat glutenin using the protein-processing system of rice. The gene we transferred to rice was slightly different from that reported by Araki et al. (2008), since we used 8.2 kb of the genomic DNA of Glu-1Dx5 that includes the native promoter of the Glu-1Dx5 gene. The present results showed the expected activity of the wheat promoter for Glu-1Dx5 gene in rice endosperm, although endosperm specificity was not clear. Success in introducing this valuable gene (Glu-1Dx5) into rice cultivar will help us improve rice flour quality traits. These promising transgenic rice plants will be examined further and their benefits will be assessed in future rice breeding programs.

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