Development of Simple Functional Markers for Low Glutelin Content Gene 1 (Lgc1) in Rice (Oryza sativa)

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Abstract: Rice with low glutelin content is suitable as functional food for patients affected with kidney failure. Low glutelin-content gene Lgc1 in rice had a 3.5-kb deletion between two highly similar glutelin genes GluB4 and GluB5, which was located on the short arm of chromosome 2. To improve the selection efficiency in low glutelin-content rice breeding, we developed two molecular markers to detect the low glutelin-content gene Lgc1, which were designated as InDel-Lgc1-1 and InDel-Lgc1-2, respectively. A double PCR detection indicated that combined use of the two markers could distinguish the genotypes of Lgc1 from different rice varieties easily. Therefore, as a simple and low-cost technique, it could be used widely to identify different varieties with Lgc1 gene and applied in marker-assisted selection of low glutelin-content rice.

Key words: Oryza sativa; low glutelin-content gene; Lgc1 gene; functional marker; double PCR

As one of the storage substances in seed, rice endosperm protein plays an important role in daily life of human. It can be divided into four different forms: glutelin, prolamine, albumin and globulin. Glutelin is the major digestible component (60%–80%) in seed proteins, which is synthesized as a 57-kD precursor and then cleaved into a 37- to 39-kD acidic subunit and a 22- to 23-kD basic subunit in cytoplasm. The nutritional value of rice can be raised by improving its glutelin content (Shewry and Casey, 1999). However, for patients affected with kidney failure, a large amount of glutelin absorbed can lead to proteometabolism disturbance and exacerbation. To meet the special requirement of these patients, it has become a major research area in functional rice breeding to develop new low glutelin-content varieties with excellent agronomic traits (Iida et al, 1993).

Low glutelin-content mutant is an important genetic resource for breeding low glutelin rice, and its distinguishing feature is characterized by less mature glutelin (37- to 39-kDa, 22- to 23-kDa in protein body type II) and more prolamine (10 kDa, 13 kDa and 16 kDa in protein body type I) than those of normal varieties. NM67 was the first low-glutelin content mutant, which obtained by treatment with chemical reagents. Breeders in Japan developed a commercial variety LGC-1 from this mutation in rice in 1993 (Iida et al, 1993). Genetic analysis of low glutelin-content trait in LGC-1 indicated that it was controlled by a single dominant gene Lgc1, which located between RFLP markers XNpb 243 and G365 on the short arm of chromosome 2, and the genetic distances to Lgc1 were estimated to be 8.5 cM and 39.1 cM, respectively (Miyahara et al, 1996). With similar physical or chemical mutagenesis, other low glutelin-content mutants were acquired by Iida et al (1997) and Qu et al (2002). In these mutants, type-1, type-2 and type-3 were controlled by different single recessive genes glu-1, glu-2 and glu-3, respectively, and RFLP analysis also indicated that glu-1 was allelic to Lgc1, and glu-2 and glu-3 were on chromosomes 10 and 1, respectively.

With the rapid development of technology in molecular biology, the mechanism of mutation in low glutelin-content variety LGC-1 had been elucidated by Kusaba et al (2003). In LGC-1, a 3.5-kb deletion between the GluB4 and GluB5 genes, which was located with an inverted orientation and shared 99.8% nucleotide sequence identity, resulted in the production of a tail-to-tail inverted repeat of the two
GluB genes. Transcription of the fused GluB4-GluB5 gene (lgc1, accession number AB093593) produced an mRNA with sense GluB4 and antisense GluB5 sequences, which formed a hairpin structure with an intramolecular double-stranded RNA in the complementary region. This double-stranded RNA was considered to induce RNA interference against transcripts of the GluB gene subfamily, resulting in remarkable suppression of GluB protein accumulation in LGC-1.

In this study, we developed two molecular markers to detect the low glutelin-content gene Lgc1, according to a 3.5-kb nucleotide sequence deletion in low glutelin-content rice varieties. The results of PCR detection indicated that combined use of these two markers could distinguish the genotypes of Lgc1 in different rice varieties.

MATERIALS AND METHODS

Rice materials

The rice varieties used in this study were two low glutelin-content japonica varieties LGC-1 and W3660 (Lgc1 gene derived from LGC-1, Wan et al, 2004), and normal glutelin-content rice varieties: Nipponbare, Wuyunjing 3, 02428, 9311 and Nanjing11. The F1 seeds were derived from reciprocal crosses between W3660 and two rice varieties Nipponbare and 02428. Those rice materials were grown in experimental plots at the Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, China in 2009 and managed periodically as required.

Strategy of PCR primer design

The accession number P0693E08 of P1-derived artificial chromosome (PAC) clone was obtained from the basic local alignment search tool (BLAST) analysis of the nucleotide sequences in coding regions of GluB5 at the website of rice genome annotation (http://rice.plantbiology.msu.edu/), and corresponding nucleotide sequences including GluB4 and GluB5 were downloaded from the website of GenBank (www.ncbi.nlm.nih.gov/pubmed/). In the 3.5-kb deletion between the GluB4 and GluB5 genes, two pairs of primers with same annealing temperature (56ºC) and different amplified fragment lengths (881 bp and 509 bp) were designed by the Primer Premier 5.0 software (http://www.premierbiosoft.com). The sequences of primers were as follows: 5' - TTCTACAA TGAAGGCGATGC-3' and 5' - CTGGGCTTTAACGG GACT-3' for InDel-Lgc1-1; 5' - ACCGTGTATGGCA GTTT-3' and 5' - ATTCAGGGCTATCGTCT-3' for InDel-Lgc1-2 (Fig. 1).

 Extraction of storage proteins in rice seeds and assays by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Mature seeds were harvested from parents and F1. Protein extraction was performed as described by Cagampang et al (1966). Briefly, place grains with removed embryo in a mortar, and grind it into fine powder; Transfer the powder into a 1.5 mL Eppendorf tube, and add 700 μL SDS-Urea extract solution which consists of 8 mol/L Urea, 4% SDS, 5% β-mercaptoethanol, 20% glycerol and 50 mmol/L Tris-HCl buffer (pH 6.8); Finally, the powder was mixed thoroughly and incubated overnight at 25ºC. After centrifugation at 7000 r/min for 5 min, 5 μL of supernatant were loaded for SDS-PAGE. SDS-PAGE analysis followed Laemmli (1970) and gels were stained with coomassie brilliant blue R250.

Total DNA extraction from rice leaves

Genomic DNA was extracted from rice leaves...
following the method described by Dellaporta et al. (1983) and detailed steps of DNA extraction were as follows: Place 0.5–2.0 g of plant leaves in a mortar and add an excess of liquid nitrogen. When the nitrogen evaporates, grind the tissue thoroughly into fine powder and transfer it into a 1.5 mL Eppendorf tube. Add 600 μL of DNA extraction buffer (20% SDS, 1 mol/L Tris-HCl, 0.5 mol/L EDTA and 5 mol/L NaCl) and mix thoroughly. Incubate the tube at 65°C in a water bath for 30 min, and then invert several times gently. Add 1/4 volume of 5 mol/L potassium acetate, and incubate the tubes on ice for at least 30 min. Add 300–400 μL chloroform-isoamyl alcohol mixture (24:1) and oscillate on shaking table at 120 r/min for 30 min. Centrifuge at 8000–10000 r/min for 15 min at 4°C. Transfer the supernatant (400 μL) to a new tube. Add an equal volume of chloroform-isoamyl alcohol mixture (24:1) and oscillate on the shaking table at 80–90 r/min for 30 min. Centrifuge at 8000 r/min for 15 min at 4°C, and transfer the supernatant (400 μL) to a new tube. Precipitate the DNA by adding twice the volume of ice-cold ethanol and invert several times gently. Centrifuge at 12 000×g for 6 min at 4°C. Discard the ethanol and wash the DNA pellet with 200 μL ice-cold 70% ethanol. Air dry and dissolve the DNA in 100–200 μL of TE buffer and store at –20°C.

PCR amplification and agarose gel electrophoresis

A 20 μL of PCR reaction mixture included DNA (10 ng/μL) 2.0 μL, 10×Buffer 2.0 μL, MgCl2 (25 mmol/L) 1.2 μL, primers InDel-Lgc1-1 1.0 μL (4 pmol/μL), primers InDel-Lgc1-2 1.0 μL (4 pmol/μL), dNTPs 0.4 μL (2.5 mmol/L), Taq 0.2 μL (5 U/μL) and ddH2O 12.2 μL. PCR reaction was performed as described by Chen et al (1997), and only the annealing temperature (56°C) was modified. Amplification products were visualized in 1.2% gels and stained with ethidium bromide.

RESULTS

SDS-PAGE analysis of storage proteins in different rice seeds

The protein profile analysis showed that low glutelin-content varieties LGC-1 and W3660 had more prolamine (10 kDa, 13 kDa and 16 kDa), less acidic subunits (37- to 39-kDa) and basic subunits of glutelin (22- to 23-kDa), compared with normal rice varieties Nipponbare, Wuyujing 3, 02428, 9311 and Nanjing 11. From the protein bands in four F1 combination seeds derived from reciprocal crosses between W3660 and two rice varieties, Nipponbare, Wuyujing 3, 02428, 9311 and Nanjing 11, there was no significant difference observed in Fig. 2. It was clearly indicated that the low glutelin-content trait was dominant inheritance and no gene dosage effect in rice endosperm. This result was completely consistent with the conclusion reported by Miyahara (1999).

Fig. 2. SDS-PAGE analysis of storage proteins in different rice parents and F1 combination seeds.

M, Protein marker, 14.4–116.0 kDa; Lanes 1 to 7, LGC-1, W3660, Nipponbare, Wuyujing 3, 02428, 9311 and Nanjing 11, respectively; Lanes 8 to 11, W3660/Nipponbare, Nipponbare/W3660, W3660/02428 and 02428/W3660, respectively.
PCR detection with molecular marker InDel-Lgc1-1

Total eleven rice materials including seven varieties and four F1 combinations were used for PCR detection with the designed marker InDel-Lgc1-1 (Fig. 3). The electrophoresis detection showed that a DNA fragment about 881 bp could be stably amplified in LGC-1, W3660 and four F1 plants, but no fragment was observed in Nipponbare, Wuyujing 3, 02428, 9311 and Nanjing 11. The above results did not coincide with the prospection. Because there was no 3.5-kb deletion between GluB4 and GluB5 genes in normal glutelin-content rice, a DNA fragment about 4381 bp should be amplified in these varieties. However, this band was never present in this experiment, and the reason might be due to the limited ability of Taq DNA polymerase to amplify large fragment of DNA sequences. Therefore, InDel-Lgc1-1 was a dominant marker with similar results identified by protein electrophoresis.

PCR detection with molecular marker InDel-Lgc1-2

In order to better distinguish homozygous and heterozygous genotypes of Lgc1, another pair of primers was synthesized in the 3.5-kb deletion between the GluB4 and GluB5 genes. The results of PCR detection indicated that a DNA fragment about 509 bp was amplified in normal varieties and F1 plants, but no fragment was amplified from the low glutelin-content rice varieties LGC-1 and W3660 (Fig. 4). Therefore, InDel-Lgc1-2 could be regarded as an effective complement for InDel-Lgc1-1 to distinguish the different genotypes of Lgc1.

Double PCR detection with molecular markers InDel-Lgc1-1 and InDel-Lgc1-2

In consideration of InDel-Lgc1-1 and InDel-Lgc1-2 with similar annealing temperature and different amplified fragment lengths, we tried to add two pairs of primers into a single reaction tube and distinguish three genotypes of Lgc1 by only one-step PCR method. The results of electrophoresis demonstrated that the PCR products with homozygous genotype of Lgc1 in LGC-1 and W3660 was amplified only one band about 881 bp and those with normal glutelin-content genotype showed a single band about 509 bp in the gel. In four F1 plants, it could be amplified two bands about 881 bp and 509 bp, respectively (Fig. 5). The electrophoresis bands were identical with the expected results.

DISCUSSION

Kidney failure is a clinically common and refractory disease and it has become a world-wide public health problem with the change of lifestyle and dietary structure in recent years. In the US, 9.6% of non-institutionalized adults are estimated to have chronic kidney disease (CKD), and studies from Europe, Australia and Asia also confirm the high prevalence of this disease (Levey et al, 2007). These patients are restricted to ingesting the low-protein diet because high-protein foods always lead to a serious disturbance of protein metabolism. However, special low-protein products for kidney disease are more expensive and have fewer categories compared with...
ordinary food in markets, undoubtedly it will create more financial and spiritual burden on patients. Therefore, rice with low glutelin content is considered as a cost-effective choice for diet therapy in patients with kidney failure (Mochizuki and Hara, 2000). With the growing demand on low glutelin-content rice, it has become a new focus in functional rice breeding to develop commercial varieties of low glutelin content.

In traditional breeding, the trait of low glutelin content can be identified by SDS-PAGE analysis after seed maturity. However, there are numerous problems in this process. It is not only influenced by growth and developmental stages of rice, but also difficult to pyramid many excellent genes in one variety. With the development of modern biology, molecular marker-assisted selection (MAS) has played an important role in rice breeding to improve yield, quality and resistance (Liang et al, 2004; Liu et al, 2006; Deng et al, 2007; Liu et al, 2008; Chen et al, 2009; Wang et al, 2009). However, it is surprising that only a few varieties or lines have been bred by MAS so far. The reason can be explained in two aspects. On one hand, the genetic distance between gene and marker is too far to effectively select in rice breeding, and on the other hand, the basic research is not closely correlated with breeding practice. It is well known that populations used for gene mapping are always derived from indica-japonica crosses, but markers in these populations show less polymorphism in breeding population and can not be applied in rice improvement. Therefore, the best method for resolving this problem is to develop functional markers based on the sequence variation of target gene.

In this study, we developed two molecular markers to detect the low glutelin-content gene \textit{Lgc1} based on a 3.5-kb nucleotide sequences deletion in low glutelin-content rice. Theoretically, InDel-\textit{Lgc1}-1 was a co-dominant marker, but special Taq DNA polymerase for amplifying large fragment DNA sequences must be used in experiment. Undoubtedly it would increase the test cost and reduce the possibility in practical application. To distinguish genotypes of \textit{Lgc1} more effectively, another marker designated as InDel-\textit{Lgc1}-2 was synthesized as a complement for InDel-\textit{Lgc1}-1. In breeding practice, we adopted the method with double PCR detection and obtained satisfactory results. Therefore, as a simple and low-cost technique, it could be used widely to identify different varieties with \textit{Lgc1} gene and applied in marker-assisted selection of low glutelin-content rice.

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