Research Report

Design and Validation of Two InDel Markers for Low Glutelin Content (Lgc1) Gene in Rice (Oryza sativa L.)

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Abstract

Rice with low glutelin content is quite effective as a kind of functional food for patients with kidney disease. It is of great importance to develop commercial rice varieties of low glutelin content. In recent years, Lgc1 gene, as an excellent genetic resource for rice varieties with low glutelin content, has been given more and more attention by breeders in functional rice breeding. To improve the precision of marker-assisted selection for Lgc1 gene in rice, we designed two pairs of functional markers for Lgc1 gene based on a 3.5 kb deletion fragment between two highly similar glutelin genes GluB4 and GluB5 in two rice mutants. Two InDel markers were designated and named In-Del-Lgc1-A and InDel-Lgc1-B, respectively. These markers were used to analyze 13 rice varieties and the F2 segregation population derived from the cross of the low glutelin content rice variety W3660 and normal variety Nanjing46. The PCR results showed that according to the band types the homozygous genotype with low glutelin content, the heterozygous genotype, and the homozygous genotype with normal glutelin content could be clearly distinguished, which was completely consistent with the results of the protein analysis. Thus, these two InDel markers could be used to distinguish rice varieties with Lgc1 gene, and can also be used in marker-assisted selection.

Keywords: Rice; Low glutelin; Lgc1 gene; InDel marker; Marker-assisted selection

Background

Glutelin is the major storage protein and accounts for 80% of the total endosperm protein in the rice grain (Jiao et al., 2008; Liu et al., 2008). Glutelin can be easily digested and absorbed by human, which indicates that increasing glutelin content has served to improve nutritional value of rice. However, for people suffering from kidney disease and diabetes, substantial absorption of glutelin may lead to deterioration of their disease condition (Mochizuki and Hara, 2000). Therefore, to meet the special requirement of these patients for the low content of protein foods, developing low glutelin-content varieties has become an important direction in current rice breeding.

Iida et al (1993) obtained a mutant material NM67 by treating the rice seeds of “Nihonmasari” with chemical mutagen ethyleneimine and using SDS-PAGE method, and the mutant NM67 showed a significant lower content of glutelin than its original parent. Then, they bred the first low glutelin-content rice variety named LGC-1 by back-crossing between NM67 and the original cultivar Nihonmasari. Since then, the mutant LGC-1 with low glutelin-content gene Lgc1 was widely used by rice breeders as one of the parents in new variety breeding of low glutelin-content. For example, Fukuoka (1996) developed a low glutelin-content rice variety “Nishikaze 231” by crossing between NM67 and japonica rice variety “Yumehikari”. Wan et al (2004) bred early matured japonica variety W3660 with low glutelin content by crossing LGC-1 with Japanese cultivar Koshihikari, and then backcrossed with Koshihikari as the recurrent parent. By the pedigree method, Nishimura (2005) developed two excellent eating quality rice varieties with low glutelin-content, LGC-Katsu and
LGC-Jun, by crossing LGC-1 with Koshihikari.

With the deepening of the research on molecular biology of rice, the mechanism of mutation in rice variety LGC-1 has been elucidated. Genetic mechanism of LGC-1 was first investigated by Miyahara (1999), and it was found that the low-glutelin trait of LGC-1 was controlled by a single dominant gene Lgc1. With RFLP molecular markers, the gene was mapped between the markers XNpb-243 and G365 on the short arm of chromosome 2. Further research by Kusaba et al. (2003) showed that there was a 3.5 kb deletion fragment between two highly similar glutelin genes GluB4 and GluB5 in LGC-1. Because of the deletion, transcription of the GluB4 and GluB5 formed a hairpin structure with an intramolecular double-stranded RNA in the complementary region. Then, the double-stranded RNA induced RNA interference against transcripts of the GluB gene subfamily, resulting in conspicuous suppression of GluB protein accumulation in LGC-1.

At present, the gene Lgc1 has become an important breeding resource in low glutelin content of functional rice, and mutation mechanism of Lgc1 is clear. According to a 3.5 kb deletion fragment in the low glutelin-content rice, we attempted to develop molecular markers of the target gene. In this way, the efficiency of gene selection can obviously be improved.

1 Result and Analysis
1.1 Genetic analysis of low glutelin content in F2 of W3660/Nanjing 46

The protein profile analysis showed that W3660 had dense bands of prolamine (13 kD), thin bands of acidic subunits (37–39 kD) and basic subunits (22–23 kD) of the mature glutelin, compared with normal rice variety Nanjing 46. In 303 F2 seeds of W3660/Nanjing 46, 217 of low glutelin and 86 of normal individuals, the segregation of types showed a good fit to a 3:1 ratio (χ²=1.673), which obviously indicated that the low glutelin-content trait was controlled by a single dominant gene. The result was completely consistent with the conclusion reported by Miyahara (1999); Partial individual F2 seeds by SDS-PAGE were shown in Figure 1, which showed that 2, 3, 6, 11, 14, 15 individuals were normal and the others were mutant in glutelin character.

![Figure 1](http://rgg.sophiapublisher.com)

Figure 1 SDS-PAGE analysis of total protein in partial individual plants in F2 of W3660/Nanjing46

Note: M: Protein marker; P1: W3660; P2: Nanjing46; 1~20: Individual plants of F2

1.2 Design and validation of two indel markers based on gene Lgc1

Compared with the normal rice, research confirmed that there was a 3.5 kb deletion fragment between GluB4 and GluB5 in Lgc1 (Kusaba et al., 2003). According to the fact, two InDel markers, named InDel-Lgc1-A and InDel-Lgc1-B, were designed by the Primer Premier 5.0 software in the study. The sequences of primers were as follows: 5'-AAATGTATGGTCGCTCAATCG-3' and 5'-TGTGCAAGGGAGGAAAGATAGC-3' for InDel-Lgc1-A; 5'-AAATGTATGGTCGCTCAATCG-3' and 5'-CATCAGTGTTGGGAATGTCG-3' for InDel-Lgc1-B. In the above two pairs of primers, the 5'-AAATGTATGGTCGCTCAATCG-3' was a common primer sequence obviously (Figure 2).

The electrophoresis detection showed that all of them amplified only one band, 4 328 bp or 828 bp, lacking of hybrid type, presenting the characteristics of dominant marker. In order to make the heterozygous genotype to perform, we further developed a new primer in the absence of fragment. It was used with one of InDel-Lgc1-A to form a pair primers InDel-Lgc1-B, which amplified fragment size was 1 573 bp. Therefore, the detection was carried out with three primers to form two pairs of markers in the same PCR reaction system. The results showed that the heterozygous type appeared just as what we expected. In other words, products amplified in F2 can be divided into three band types: namely low glutelin Lgc1 parent W3660 (828 bp), normal parent Nanjing 46 (1 573 bp) and heterozygous types (828 bp and 1 573 bp) (Figure 3). In the F2 population, PCR results indicated that a 1:2:1 segregation ratio (78 mutants:...
139 heterozygotes: 86 normal, \( \chi^2=0.828 \) without segregation distortion. It was further evidence that the low glutelin trait was controlled by a dominant gene. And the protein and molecular markers amplification phenotype were consistent highly.

![Diagram of primer sequences and deleted fragment in Lgc1](image)

**Figure 2** Locations of the primer sequences and the deleted fragment in Lgc1

Note: The black box: the exon in GluB-4 and GluB-5; The dotted line: a 3.5 kb deletion between GluB-4 and GluB-5; The Single and double arrowheads denote the direction of gene transcription and the region of PCR primers designed, respectively.

![PCR products amplified from partial individual plants of F2](image)

**Figure 3** PCR products amplified from partial individual plants of F2 (W3660/Nanjing46) with two InDel markers

Note: M: DNA Marker; P1: W3660; P2: Nanjing46; 1~20: Individual plants of F2

1.3 Detection of two InDel markers in normal glutelin content rice varieties

In order to further validate the detection accuracy of two InDel markers for rice varieties from different areas, we used 12 rice varieties in this study, including the W3660 with gene Lgc1, by using PCR validation. The results of electrophoresis of PCR showed that 12 normal rice varieties with 1 573 bp and W3660 with 828 bp were amplified (Figure 4). These results were fully affirmed that the two InDel markers can be used to distinguish whether containing Lgc1 gene or not in rice varieties.

2 Discussion

Due to renal failure in protein metabolism, patients with diabetes or kidney disease cannot eat rice with an absorbable-protein content of more than 4%. Obviously, rice, as an important food crop, is not only staple food but also a health diet for people. It was first time that Japanese breeders developed low glutelin functional rice variety LGC-1. Then it was widely cultivated for test since 1994 and used in clinical trials for kidney disease patients. Results showed that the patient’s condition improved significantly. For this reason, LGC-1 was considered to be very effective in patients with nephropathy (especially rice as the staple food) diet supplements (Mochizuki and Hara, 2000).

Cultivation of functional rice varieties with low glutelin is useful. On the one hand, it is a good opportunity for the breeders to expand the breeding direction. On the other hand, it also brings a tremendous benefit and gospel to kidney patients. As the demand for low glutelin rice is growing, breeding of new low glutelin rice varieties to acclimate different ecological areas has become a new hot spot. In recent years, although some low glutelin materials were reported (Iida et al., 1997), the Lgc1 gene is still very popular for breeders and consumers. Consequently, how to identify Lgc1 gene more accurately, quickly and simply has become an urgent problem for low glutelin breeding in rice. With the development of biotechnology, molecular marker-assisted selection has become an important supplementary means for breeding. Its polymorphism directly reveals the differences in genomic DNA. Currently, many gene markers linked were reported, but they showed
less polymorphism in breeding population and couldn't be applied in rice improvement (Andersen and Lübberstedt, 2003). The best method for resolving this problem is to develop functional markers. They are superior to random DNA markers such as RFLPs, SSRs and AFLPs owing to complete linkage with trait locus alleles. In other words, functional marker based on the sequence variation of target gene is the guarantee of molecular marker assisted selection (Bradbury et al., 2005; Wang et al., 2009).

In this study, according to a 3.5 kb nucleotide sequences deletion in low glutelin content rice, we firstly developed marker In-Del-Lgc1-A, which amplified 4328 bp in normal plants and 828 bp in mutant containing \textit{Lgc1}. By using this marker, we detected F2 population of W3660/Nanjing 46. Unfortunately, whatever plant appeared only one band, namely 4328 bp or 828 bp, were all lack of heterozygous. The results were obviously inconsistent with theory. According to the situation, we thought that allelic fragment amplified in heterozygote differed so much, resulting in small fragments obtained significant advantage of amplification; In contrast, large fragment amplification efficiency was low, and so it was difficult to detect. Two types of plant DNA were mixed together and amplified for further confirmation of this idea, and the large fragments were still not detected. As can be seen, our idea was correct. To shorten the gap of the amplified fragment length, making the heterozygous genotype to be reflected, we further have designed a new primer, and with one of primer from In-Del-Lgc1-A formed a new marker In-Del-Lgc1-B, which amplified 1573 bp. So far, three primers consist of two markers in the same reaction system to detect \textit{Lgc1}. Using these two markers, all individuals in F2 were effective to distinguish. Because of the competition effect, normal genome could only amplify 1573 bp but not 4328 bp, anyway, it was not affected by functional marker detection. Doubtless, they could be used in marker-assisted selection of low glutelin-content rice. In addition, to guarantee the phenotype and genotype of correspondence, F2 population used half-grain method, half-grain to be cultivated seedling and another to be done for total protein SDS-PAGE. Our previous reports and experiments revealed that half-grain seedlings were weak and vulnerable to disease, and even dead early. For the reason, it was difficult to get enough leaves for the test. Here, put half-grain was disinfected into culture medium containing 0.5\% Yi Peilong bactericide, and obtained a high of 95\% plant survival rate without microbial contamination in the process of cultivating seedling (three weeks). It also provided an important reference for half-grain of cultivating seedling in the future.

3 Materials and Methods

3.1 Tested materials

With low glutelin gene \textit{Lgc1} of rice variety W3660, it was based on low glutelin variety LGC-1 as female parent and Japanese main cultivar Koshihikari hybridization, and the latter was as the recurrent parent continuously backcrossing. The rice variety planted in Jiangsu has a good yield and adaptability. 12 normal gluten-content rice cultivars or lines including Kanto 194 (Japonica rice variety, Crop Research Institute of Ibaraki, Kanto region of Japan), Nipponbare (Japonica rice variety, Aichi comprehensive agricultural test site, Japan), Aichi 106 (Japonica rice variety, Aichi comprehensive agricultural test site, Japan), Nanjing 44 (Early-maturing late-season japonica rice cultivar, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences), Ning 5 055 (Early-maturing late-season japonica rice line, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences), Nanjing 11 (Conventional indica rice cultivar, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences), Wuyunjing 7 (Early-maturing late-season japonica rice cultivar, Wujin rice and wheat breeding field, Changzhou of Jiangsu), Ningjing 3 (Early-maturing late-season japonica rice cultivar, College of Agriculture, Nanjing Agricultural University), Huajing 3 (Late maturity japonica rice varietie, Jiangsu Dahua Seed Limited Company), Yandao 9 (Late maturity japonica rice varietie, Yanhai Institute of Agricultural Science, Jiangsu). F2 segregating population, which was from the cross Nanjing 46×W3660 (female parent).

3.2 Materials planting

To extract DNA, the materials were planted in our
laboratory in November 2009. F2 population was used half-grain method. In other words, they were crosscut, and divided into roughly two parts of the same length. One half-grain with embryo was planted in MS medium. (specific methods: Firstly, Shanghai Jiafeng MS powder medium with 41.5 g/L ratio was dissolved in water, added 0.5% bactericide of Yi Peilong, after 121°C high temperature sterilization 20 min, and then put into ZP17-440 jar to reserve. Secondly, half-grain was disinfected, 75% alcohol for 20 minutes, 0.1% mercuric chloride for 15 minutes, after distilled water washing three times. Finally, put it into the jar with MS medium. Placed into incubator with 30°C for continuous light culture). The other part was used for total protein SDS-PAGE analysis. Normal rice seeds were directly cultured with water in culture dish.

3.3 Extraction of total proteins in rice seeds and phenotype analysis by SDS-PAGE
Half-grain of F2 without embryo and normal rice seeds were used for total protein extraction. Protein extraction was performed as described by Jiang Shaomei (2003) method, slightly changed. Half-grain was grinded into fine powder, and placed into a 1.5 mL Eppendorf tube, to which was added 150 µL Tris-HCl buffer, pH 6.8). The tube was vortexed and incubated overnight at room temperature (25°C). Whole grain protein extraction used the same methods as half-grain, but double reagent was added. After centrifugation at 10 000 r/min for 10 min, 10 µL of supernatant was loaded onto an SDS-PAGE gel (15% polyacrylamide gel for separation, 7.5% polyacrylamide gel for stacking). Following electrophoresis, gels were stained with CBB-R250.

3.4 DNA extraction, PCR amplification and electrophoresis
Tender leaves from rice were taken about 6~8 cm long. Then genomic DNA was extracted following the method described by Dellaporta et al (1983). The two InDel markers, which were designed, analysis were performed by PCR. Each 25 µL PCR reaction mixture contained TaKaRa 2×PremixLA Taq 12.5 µL, the forward and reverse primers 1.5 µL (4 pmol/µL), respectively; DNA 3.0 µL (50~100 ng/µL) and ddH2O 6.5 µL. The amplification procedure is as follows: 32 cycles of 10 s denaturation at 98°C, 30 s annealing at 56°C and 270 s extension at 72°C, followed by a final 10 min extension at 72°C. Amplification products were electrophoresed on 1% agarose gels and stained with ethidium bromide (EB).

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