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ORIGINAL PAPER

Development of PCR markers to detect the *glb1* and *Lgc1* mutations for the production of low easy-to-digest protein rice varieties

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Abstract Limiting the ingestion of protein is the fundamental idea in the diet therapy for patients with chronic renal failure. Two mutations involved in the content of major rice storage proteins useful for developing low easy-to-digest protein rice variety have been isolated. The *glb1* mutation causes the deficiency of α-globulin, and the *Lgc1* mutation reduces the glutelin content. By combining the *glb1* and the *Lgc1* mutations, it is possible to reduce the easy-to-digest protein content by approximately 50%. The *Lgc1* mutation has been shown to be caused by a 3.5-kb deletion between the glutelin structural genes, *GluB4* and *GluB5*, while the molecular basis of *glb1* mutation has been less understood. PCR analysis of the *glb1* mutation revealed a 62.8-kb deletion, including the structural gene of α-globulin. Based on these lines of information, we generated PCR markers that make it possible to detect the *glb1* and *Lgc1* mutations. Using those PCR markers, we genotyped F_2 plants segregating for the *glb1* mutation and the *Lgc1* mutation and confirmed the consistency of genotype and phenotype. Because the PCR marker sets can distinguish heterozygotes, they will be very useful in developing new varieties of low easy-to-digest protein rice.

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Introduction

In chronic renal failure, to reduce the burden on the kidneys, it is necessary to provide patients with therapy based on a low-protein diet (Fouque et al. 2000). Rice, which is a staple food for over half of the world's population, contains approximately 9% protein (Tanaka 1983). For patients with kidney disease, it is important to reduce this protein content. It is possible to remove protein by processing the rice with lactobacilli, or to use starch rice. But both of these methods are expensive and also result in loss of flavor. It would therefore appear desirable to breed new varieties of low-protein rice. Glutelin, α-globulin and prolamin are storage proteins accumulated in the endosperm of rice. Prolamin, which is accumulated in protein body I (PB-I), is not readily digested by humans, but glutelin and α-globulin, accumulated in protein body II (PB-II), are easy-to-digest proteins (Ogawa et al. 1987). To develop low-protein rice strains, it is therefore important to reduce the content of glutelin and α-globulin.

NM67 has been identified as a mutant with reduced glutelin content. NM67 carries a dominant mutation, *Low glutelin content1* (*Lgc1*), which was induced by treatment with ethylenimine, resulting in reduced glutelin content and increased prolamin content (Iida et al. 1993). "LGC1", a low-glutelin variety developed using the *Lgc1* mutation, is now being cultivated in Japan, and its suitability as a staple

food for patients with chronic renal failure has been confirmed (Mochizuki and Hara 2000). "LGC1" shows about 30% reduction in easy-to-digest protein content in comparison with ordinary rice varieties. In addition to "LGC1", the varieties "LGC-Soft", "Shun-yo" and "Yumekanae" have also been developed by introducing the *Lgc1* mutation.

GbN-1, a recessive mutant line in which α-globulin is completely absent, has also been selected (Iida et al. 1998). "LGC1" and GbN-1 have been crossed to develop the varieties "LGC-Jun" and "LGC-Katsu", and those varieties have been cultivated (Nishimura et al. 2005). Both "LGC-Jun" and "LGC-Katsu" are double-mutant lines, with mutations conferring low-glutelin levels and the absence of α-globulin. The easy-to-digest protein content of rice from these varieties is approximately 50% lower than from ordinary varieties. Hence, the combination of these two mutations has been thought to be important for generating low-protein varieties of rice.

The *Lgc1* mutation is a 3.5-kb deletion occurring in the *GluB4/GluB5* region (Kusaba et al. 2003). It has been found that RNA interference is induced in the synthesis of double-stranded (hairpin) mRNA, and glutelin content is reduced. However, the mutation that gave rise to GbN-1 has not yet been clearly elucidated. The rice genome has been fully sequenced (International Rice Genome Sequencing Project 2005), and the nucleotide sequence of cDNA for α-globulin has been determined (Shorrosh et al. 1992; Krishnan and Pueppke 1993). With regard to GbN-1 mutants, this means that if a mutation has occurred in the α-globulin gene or in a neighboring region, that mutation can be relatively easily detected.

We identified mutations occurring in the mutant strain GbN-1, in which α-globulin is absent. Our result showed a 62.8-kb deletion in the region including the α-globulin gene in GbN-1. We named this the "*glb1* mutation", and generated a PCR marker to detect the *glb1* mutation, enabling genotyping of the *glb1* locus. We also generated a PCR marker that similarly enables genotyping of the *Lgc1* mutation, conferring reduced glutelin content. The PCR markers that we generated in the course of this research make it possible to genotype for *glb1* and *Lgc1*, two important mutations for the breeding of low-protein varieties of rice.

Materials and methods

Plant materials

The GbN-1 mutant has been obtained from rice (*Oryza sativa* L.) by irradiating the variety "Koshihikari" with gamma rays (Iida et al. 1998). "LGC1" has been developed from a backcross between NM67 mutant and "Nihonmasari" (Iida et al. 1993). "LGC-Jun" has been developed from a cross between "LGC1" and the GbN-1 mutant (Nishimura et al. 2005).

Examination of deletion by PCR

DNA was extracted from 100 mg of mature leaves by using DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). PCR was performed with a total volume of 20 μL, containing 20 ng of DNA, 2 μL of 10× ExTaq buffer (Takara Bio, Shiga, Japan), 1.6 μL of 2.5 mM dNTP, 10 pmol of each primer, and 0.5 U of Taq DNA polymerase (ExTaq, Takara Bio). The PCR conditions were 30 s of denaturation at 94°C, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 40 s.

Thermal asymmetric interlaced PCR and nucleotide sequencing

Thermal asymmetric interlaced PCR (TAIL-PCR) was performed as described by Liu et al. (1995) using Taq DNA polymerase (rTaq, Takara Bio), 43-1, 43-2 and 43-3 as specific primers (Table 1) and AD2: 5′-GTNCGA(G/C)(A/T)CANA(A/T)GTT-3′ as a degenerate primer (Miyao et al. 1998). The PCR product was purified with QIAquick Gel Extraction Kit (Qiagen) and was directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan). The comparison between the resulting nucleotide sequence and a "Nipponbare" genomic clone was made using the Sequencher program (Hitachi Software, Tokyo, Japan).

Table 1 Nucleotide sequences of the primers used in this study

Primer	Sequence (5′–3′)
GLBw-F	CAATGGCTAGCAAGGTCGTCT
GLBw-R	CAAGCCAGCTAAGCCTAGTAC
−43 kb-F	TAGAACTGTCCCAATTGCTC
−43 kb-R	CTGTTCCTCTGTTCTCATTC
−42 kb-F	CTAAATTAATTCCTGACCCTTG
−42 kb-R	ACGGTTCATTAGCACGTGAT
43-1	GGCAAAAAATGCACTTGGTCATTTC
43-2	TTTCTGATTTTAGCTGCACATGCAT
43-3	CTGTTGGGGAAGTTCTGCACTAAC
AD-2	GTNCGA(G/C)(A/T)CANA(A/T)GTT
del-R	CACTGGTGGTGTACTACCAT
GLBd-F2	GAGAACAGAGGAACAGAGATGTT
GLBd-R2	TGAGGTGTCCTCTCCTCTTTTAG
LGCd-F2	CGGGTAAAGAATGGCCTTAAAC
LGCd-R2	ATTTGGTCATACTAGTCTAGTT
LGCw-F	GCAAGCACGAAGCCTTAAGA
LGCw-R	TGAGTCCACAGTTGATTGCT

Genotyping the *glb1* locus and the *Lgc1* locus

We used a DNeasy Plant Mini Kit (Qiagen) to extract genomic DNA of LGC-Jun and Nipponbare of rice. We combined equivalent amounts of DNA from Nipponbare and LCG-Jun to obtain a DNA mixture. Multiplex PCR was performed with 20 μL mixture containing 20 ng of DNA, 2 μL of 10× ExTaq buffer (Takara Bio), 1.6 μL of dNTP, 10 pmol of each primer and 0.5 U of Taq DNA polymerase (ExTaq, Takara Bio). The PCR conditions were 30 s of denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 40 s. For genotyping the *glb1* locus, we used the primers GLBd-F2, GLBd-R2, GLBw-F and GLBw-R, and added PCRx enhancer (Invitrogen, Tokyo, Japan) at 2× concentration. For genotyping the *Lgc1* locus, we used the primers LGCd-F2, LGCd-R2, LGCw-F and LGCw-R.

Phenotyping of F_2 seeds by SDS-PAGE and genotyping of the *glb1* locus and the *Lgc1* locus

LGC-Jun was crossed with IR36, and F_2 seeds were obtained. Following the method described by Iida et al. (1993), we extracted the proteins from each F_2 half seed and performed SDS-PAGE analysis. The phenotype was determined after Coomassie Brilliant Blue staining. The remaining half seeds containing the germ were cultivated in MS nutrient medium (Murashige and Skoog 1962), and DNA was extracted from mature leaves and used as a template for PCR.

Result

Analysis of the GbN-1 mutation

We used PCR to reveal the mutation causing GbN-1. When we performed PCR using primers that amplify the coding region in the α-globulin gene (GLBw-F and GLBw-R, Table 1), no band was detected, indicating that a deletion of the coding region occurred in the GbN-1 mutant (Fig. 1a). In order to investigate the size of the deletion, we generated primers having sequences upstream of the α-globulin gene and performed PCR. When we used primers that amplify about 4, 10, 20, 22, 28, 30, 32 and 37-kb upstream region of the α-globulin gene, no amplification of each DNA fragment was observed (data not shown). Similarly, when we used primers that amplify a 42-kb upstream region of α-globulin gene (−42 kb-F and −42 kb-R), no band was detected (Fig. 1a). However, a band was detected when we used primers amplifying a 43-kb upstream region (−43 kb-F and −43 kb-R), indicating that the region from the α-globulin gene to the 42-kb upstream sequence was deleted in the GbN-1 mutant (Fig. 1a). In order to determine the deletion size in greater detail, we used TAIL-PCR to amplify the flanking region of the 43-kb upstream of α-globulin gene. TAIL-PCR yielded a PCR product of about 1.5 kb (data not shown). We determined the nucleotide sequences of a 300 bp of the PCR product, and a BLAST search showed the 1–37 bp to be identical to 77,984–78,020 in the Nipponbare genomic clone (Accession No. AC113332), and the remaining 38–300 bp to be identical to 14928–15190 in AC113332 (Fig. 1b). When we used a primer del-R for the 14,928–15,190 region of AC113332 and −43 kb-F in PCR, a band was detected (Fig. 1a). This result strongly suggests that a deletion of a region corresponding to 62,793 bp in Nipponbare took place in GbN-1 mutant, and that this deletion caused a loss of α-globulin protein (Fig. 1c). We named the 62.8-kb deletion induced in the GbN-1 mutant as the "*glb1* mutation".

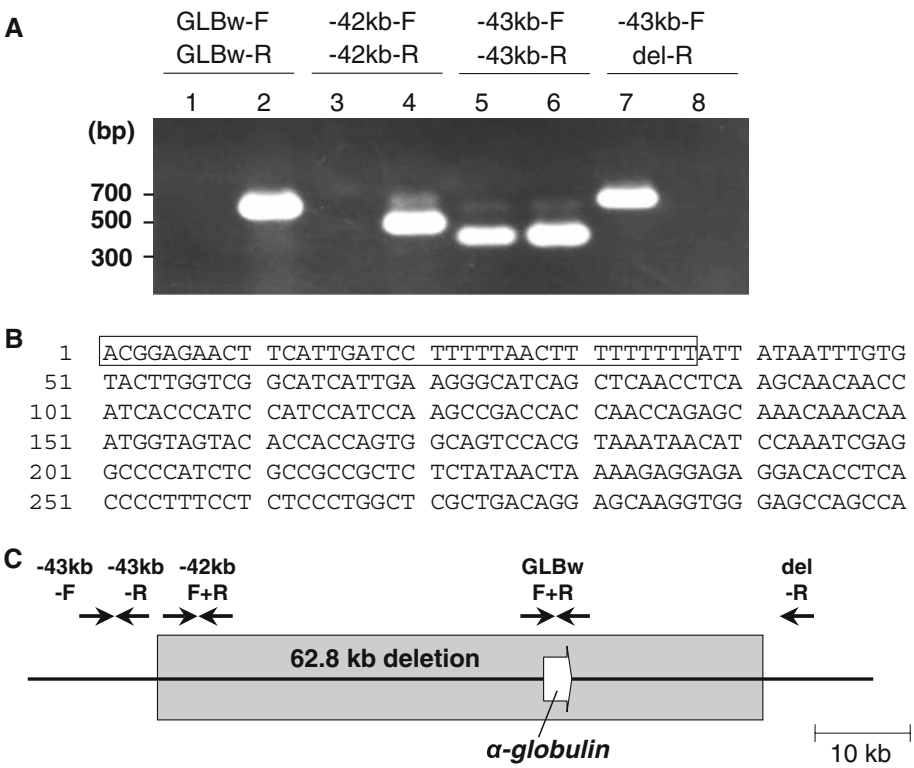
Markers for detecting the *glb1* mutation and *Lgc1* mutation

To detect the *glb1* mutation, we produced primers GLBd-F2 and GLBd-R2 for a deletion-flanking sequence (Fig. 2a). The primers GLBw-F and GLBw-R, which amplify the coding region of the α-globulin gene, can serve as PCR markers for the α-globulin gene (Fig. 2a). LGC-Jun is homozygous for the *glb1* mutation (Nishimura et al. 2005), and Nipponbare is homozygous for wild type (WT) α-globulin allele. When we performed multiplex PCR using templates of DNA from each of these varieties and a mixture of DNA from both varieties, with GLBd-F2, GLBd-R2, GLBw-F and GLBw-R as primers, a band of 304 bp was detected for templates using the LGC-Jun DNA and the mixed LCG-Jun/Nipponbare DNA (Fig. 2b). A band of 576 bp was detected for templates using the Nipponbare DNA and the mixed DNA (Fig. 2b).

The *Lgc1* mutation is a 3.5-kb deletion occurring in the *GluB4/GluB5* region (Kusaba et al. 2003). In order to prepare a marker that enables the detection of the *Lgc1* mutation, we produced primers LGCd-F2 and LGCd-R2 for a deletion-flanking sequence (Fig. 3a). LGCw-F and LGCw-R having the WT sequence at the deleted region were designed to detect the WT *Lgc1* allele (Fig. 3a). We performed multiplex PCR using LGCd-F2, LGCd-R2, LGCw-F and LGCw-R. A band of 274 bp was detected for templates using the LGC-Jun DNA and mixed DNA (Fig. 2b). A band of 625 bp was detected using the Nipponbare DNA and mixed DNA (Fig. 2b).

Genotyping of F_2 plants between LGC-Jun and IR36

We crossed LGC-Jun with the Indica-type rice variety IR36 for introducing both the *glb1* mutation and the *Lgc1* mutation into IR36. After obtaining F_2 seeds, we extracted protein from the half seeds and performed phenotype analysis



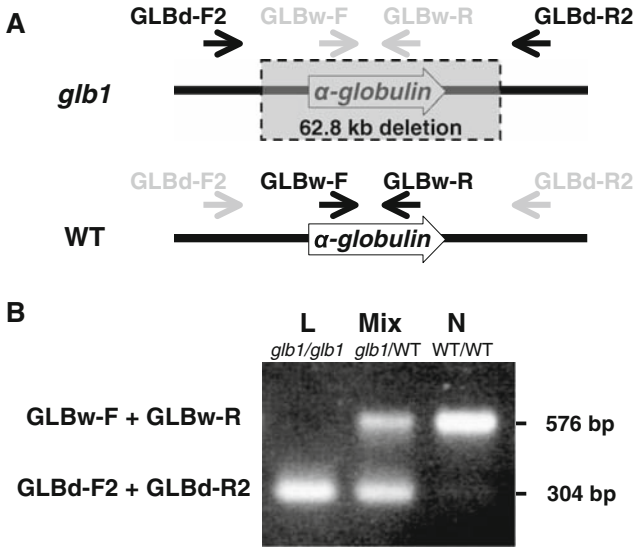


Fig. 2 Generating PCR markers for the detection of the *glb1* mutation. **a** *glb1* mutation and primer sites. The *gray box* shows the deletion site, and the *arrows* indicate primer annealing sites. *Black arrows* are used for primers associated with amplification, and *gray arrows* for those primers unrelated to amplification. **b** Genotyping for the *glb1* locus using PCR markers. The primers associated with amplification are shown at the *left*. PCR was performed using as a template the DNA from L (LGC-Jun), N (Nipponbare), and Mix (mixed DNA from LGC-Jun and Nipponbare), respectively. The genotypes for each DNA are shown above the photographs

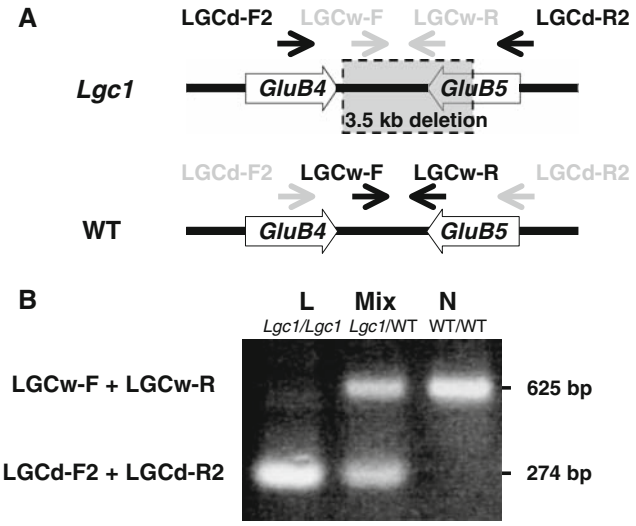


Fig. 3 Generating PCR markers for the detection of the *Lgc1* mutation. **a** *Lgc1* mutation and primer sites. The *gray box* shows the deletion site, and the *arrows* indicate primer annealing sites. *Black arrows* are used for primers associated with amplification, and *gray arrows* for those primers unrelated to amplification. **b** Genotyping for the *Lgc1* locus using PCR markers. PCR was performed using as a template the DNA from L (LGC-Jun), N (Nipponbare), and Mix (mixed DNA from LGC-Jun and Nipponbare), respectively. The genotypes for each DNA are shown above the photographs

by SDS-PAGE. In 44 F_2 seeds, phenotypic segregation of both mutations was observed by the absence of α-globulin and low glutelin content (Fig. 4).

In phenotypic segregation based on the presence or absence of α-globulin, we found eight plants to be of the *glb1* phenotype and 36 plants to be of the WT phenotype (Fig. 4). Genotyping of the eight plants that exhibited the

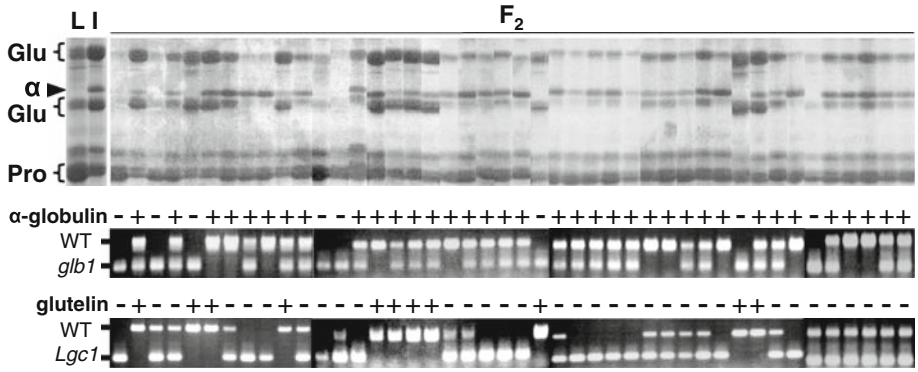


Fig. 4 Comparison of phenotyping and genotyping for *glb1* and *Lgc1* using F_2 plants from the LGC-Jun and IR36 varieties. *L*, *I* and F_2 indicate LGC-Jun, IR36 and F_2 plants from LGC-Jun and IR36 varieties, respectively. *Glu* glutelin, *α* α-globulin, *Pro* prolamin. F_2 plants exhibiting the mutant phenotype are designated "−", and F_2 plants exhibiting the WT phenotype are designated "+"

glb1 phenotype showed all 8 to be homozygous for the *glb1* mutation. Genotyping of the 36 individual plants that exhibited the WT phenotype showed 11 to be homozygous for the WT allele and 25 to be heterozygous for the *glb1* mutation and the WT, so that the phenotypic and genotypic analyses were in full agreement.

A total of 33 plants exhibited the *Lgc1* phenotype (low glutelin and high prolamin content) and 11 exhibited the WT phenotype (Fig. 4). The results of genotyping showed that all 11 plants exhibiting the WT phenotype were homozygous for the WT allele. Genotyping of the 33 plants that exhibited the *Lgc1* phenotype showed 14 to be homozygous for *Lgc1* mutation and 19 to be heterozygous, so that phenotypic and genotypic analyses were in full agreement.

We obtained F_3 seeds from the 11 F_2 plants that had been genotyped as heterozygous for the *glb1* locus and performed phenotype analysis by SDS-PAGE. Phenotypic segregation occurred for the F_3 seeds of all of the plants, confirming that the F_2 seeds were heterozygous. In the same way, phenotypic segregation was confirmed for the F_3 seeds obtained from the eight F_2 plants genotyped as heterozygous for the *Lgc1* locus (data not shown).

Discussion

The rice varieties LGC-Jun and LGC-Katsu are double mutants (carrying the *glb1* and *Lgc1* mutations) that provide reduced levels of easy-to-digest protein, approximately 50% less than from ordinary varieties (Nishimura et al. 2005). The combination of these two mutations is important for generating low-protein varieties. We successfully generated PCR markers that enabled the genotyping of the two loci. In addition, because these were gene-specific markers rather than linkage markers, there was no recombination between the marker and the mutation site. These markers will therefore be useful for genotype discrimination between the *glb1* locus and the *Lgc1* locus.

We used PCR and TAIL-PCR to determine the 62.8-kb deletion induced in rice by gamma irradiation. Irradiation induces double-stranded breaks in the DNA, giving rise not only to deletions but also to other chromosome aberrations such as inversions and translocations (Sachs et al. 2000). However, TAIL-PCR, an efficient method to amplify DNA fragments adjacent to known sequences, can be used even in cases where chromosome aberrations have occured, making it relatively easy to determine mutations. Shikazono et al. (2001), Shikazono et al. (2005) and Morita et al. (2007) used PCR and TAIL-PCR to determine irradiation-induced mutations in *Arabidopsis* and rice, so this method appears to be effective in determining mutations induced by irradiation. Although the size of the deletion in the genomic sequence of Nipponbare is equivalent to 62,793 bp, the actual size of deletion is thought to be different due to differences in genomic structures, such as insertions or deletions, because it originates from the cultivar Koshihikari. Assuming that the number of genes in the deleted region is the same for both Nipponbare and Koshihikari, ten genes (from Os05g0498800 to Os05g0500100) according to the IRGSP genome sequence build 4 (http://rapdb.dna.affrc.go.jp) are knocked out in the GbN-1 mutant. On the other hand, the visual characters of GbN-1 mutant were not different from the WT, suggesting that these genes were not related to visual characters.

The *Lgc1* mutation reduces the glutelin content. Glutelin genes consist of several subfamilies including *GluA* and *GluB* (Takaiwa et al. 1991), and the *Lgc1* mutation primarily decreases expression of the *GluB* subfamily (Kusaba et al. 2003). Therefore, if the *Lgc1* and the *glb1* mutations could be combined with a mutation deficiency in the *GluA* subfamily protein, the level of easy-to-digest protein could be reduced further. Iida et al. (1997) obtained three mutant lines deficient in glutelin subunits. We found that two of them (Type 2 and Type 3) are *GluA* subfamily mutants (Morita et al., unpublished data). We have already determined the mutation sites in Type 2 and Type 3 mutants, making it possible to generate DNA markers. The use of

these DNA markers will be useful for combining the *Lgc1*, *glb1*, Type 2 and Type 3 mutations.

Low-protein varieties have been developed in the cross between Japonica-type rice varieties, using the *glb1* and the *Lgc1* mutations (Nishimura et al. 2005). We tried to introduce the *glb1* and the *Lgc1* mutations into IR36, a variety of Indica-type rice. Phenotypic segregation observed in the F_2 plants (the low-glutelin phenotype and the α-globulin-deletion phenotype) suggests that the introduction of the *glb1* mutation and the *Lgc1* mutations could also greatly reduce the levels of easy-to-digest protein into Indica-type varieties. Because the production volume for Indica varieties is much higher than for Japonica varieties, the development of low-protein Indica-type varieties would be highly significant. In order to confirm the effectiveness of the *glb1* and *Lgc1* mutations in Indica varieties, we are currently generating a near-isogenic line of IR36 having the *glb1* and *Lgc1* mutations.

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