

Rice Mutant Lines Lacking α -Globulin

Shuichi Iida¹⁾²⁾, Kenzo Miyahara¹⁾ and Takeshi Nishio¹⁾³⁾

¹⁾ Institute of Radiation Breeding, NIAR, MAFF, Ohmiya, P. O. Box 3, Naka-gun, Ibaraki, 319-2293, Japan

²⁾ Present address: Chugoku National Agricultural Experiment Station, MAFF, Nishihukatsu, Fukuyama, 721-8514, Japan

³⁾ Present address: Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiyamachi, Aobaku, Sendai, 981-8555, Japan

To whom correspondence should be addressed.

Summary

In the screening of rice mutants having altered seed protein compositions, two mutant lines lacking α -globulin were selected from the M_2 seeds of a white panicle mutant of cv. Koshihikari irradiated with gamma-rays. The mutant character, lack of α -globulin, was found to be controlled by a single recessive gene. Northern blot analysis of endosperm RNA using the probe of α -globulin cDNA revealed that the α -globulin gene is not expressed in the α -globulin-less mutants. The mutated gene was considered to be α -globulin gene, and named *glb-1(t)*. RFLP analysis of F_2 plants between the mutant and *indica* cultivar 'Kasalath' using land marker probes suggested that *glb-1(t)* is on chromosome 5 and that the distances of *glb-1(t)* from the markers, *C246* and *XNpb366*, were 15.4cM and 30.8cM, respectively. The mutant line lacking α -globulin was expected to have a higher content of lysine in rice grains, but the lysine content of the mutant line was found not to be higher than the original cultivar. The possible use of the mutant as a breeding material is discussed.

Key Words : *Oryza sativa* L., 19kDa globulin, mutation breeding, RFLP mapping, seed protein.

Introduction

Among salt-soluble proteins in rice endosperm, the most abundant is α -globulin, which shows a single band by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Yamagata *et al.* 1982), and is separated to one large spot and one small spot by two dimensional electrophoresis (Pan and Reeck 1988). This protein occupies about 10% of the total protein in rice endosperm (Juliano 1985).

Rice endosperm globulin was separated into four components, α , β , γ , δ , from the peak fractions by gel permeation chromatography (Morita and Yoshida 1968). Pan and Reeck (1988) isolated α -globulin and indicated that the molecular weight of α -globulin estimated by gel permeation chromatography was 17,400, and that by analytical centrifuge was 16,700,

while that determined by SDS-PAGE was 26,000. Shorrosh *et al.* (1992) isolated a cDNA clone of α -globulin and estimated the molecular weight to be 19kDa from the deduced amino acid sequence. They named this globulin '19kDa globulin', but in this paper we use the name ' α -globulin', which has long been used.

Seed protein in Graminae is poor in lysine, and therefore is considered to have low nutritional value. In corn, a high lysine mutant was selected. The high lysine character of this mutant was found to be controlled by the opaque-2 gene, which reduces the content of zein, the major protein poor in lysine, and increases the content of lysine-rich glutelin (Mertz *et al.* 1964). The deduced amino acid sequence (Shorrosh *et al.* 1992) of α -globulin is devoid of lysine. Therefore, a mutant lacking α -globulin and having higher content of the other proteins instead would have a higher lysine content than the original cultivar. We selected mutant lines lacking α -globulin in the rice mutation breeding for improving endosperm protein. In this paper, we report the results of genetic analysis of the mutant lines, mapping of the mutated gene using restriction fragment length polymorphism (RFLP) analysis, and the lysine content in the endosperm of the mutant lines.

Materials and Methods

Plant materials

Dry seeds of rice (*Oryza sativa* L.) cultivar 'Koshihikari' and a white panicle mutant of 'Koshihikari' were irradiated with gamma-rays of 250Gy or 300Gy at 10Gy/h, and sown to grow M_1 plants in the field. M_2 seeds obtained from about 1,000 M_1 plants were used for the screening of mutants.

Screening of mutants

A single grain of brown rice was crushed with pliers and immersed overnight in 700 μ l SDS-urea solution, 8M urea, 4% sodium lauryl sulfate, 5% 2-mercaptoethanol, 20% glycerin and 50mM Tris-HCl buffer, pH 6.8. After centrifugation at 7,000g for 5min, 5 μ l of supernatant was subjected to SDS-PAGE. SDS-PAGE was carried out according to Laemmli (1970) on a 15% acrylamide gel.

Received June 23, 1997. Accepted December 17, 1997.

Protein extraction

Globulin-albumin, prolamine, and glutelin were sequentially extracted from the rice seeds according to Kumamaru *et al.* (1988). Polished rice (polished to 93% of brown rice in dry weight) was pulverized with a mortar and pestle. Globulin-albumin fraction was extracted from 100mg rice meal with 1.0ml of 50mM phosphate buffer (pH 6.8) containing 0.5M NaCl by shaking at 4 °C for 6h. After homogenization with a microhomogenizer (Physcotron, Niti-On Co) for 1 min, the suspension was centrifuged at 10,000 g for 15 min. This extraction was repeated three times. Prolamine was extracted from the precipitate with 1.0ml of 60% n-propanol in the same way as the globulin-albumin fraction and the extraction was repeated three times. Finally glutelin was extracted with 1.0ml of 1% lactic acid three times. After precipitation with acetone, the protein was dissolved in the SDS-urea solution and subjected to SDS-PAGE.

Northern blot analysis of α -globulin

RNA was extracted from developing seeds about 18 days after flowering according to Takaiwa *et al.* (1987). Five μ g RNA was separated by agarose gel electrophoreses with MOPS buffer containing 2.2M formaldehyde, and transferred to a nylon membrane (Hybond N, Amersham). A cDNA clone of α -globulin (EC1805, Rice Genome Program in NIAR/STAFF) and one of rice glutelin (pREE61 Takaiwa *et al.* 1987) were labeled with digoxigenin by PCR, and used as a probe for detection of mRNA of α -globulin and glutelin.

Mapping of the mutated gene by RFLP analysis

F₂ plants derived from the cross between the mutant line and an *indica* rice 'Kasalath' were used for analysis of seed protein by SDS-PAGE and of RFLP with land marker probes provided by the Rice Genome Program in NIAR/STAFF, Japan. Protein was extracted from half grains of F₂ seeds. The other half grain having embryos were sown on filter paper moistened with 0.5g/l benomyl solution in a petri dish. DNA was extracted from young plants 1 month after sowing using CTAB method (Murray and Thompson 1980). After agarose gel electrophoresis of the plant DNA digested with restriction endonucleases, the DNA was transferred to a nylon membrane (Hybond N). The DNA band was detected with the land marker probes using Dig-nonRI system (Boelinger).

RFLPs of 15 F₂ plants homozygous for the mutated gene were analyzed first for the screening of the chromosome bearing the mutated gene using more than two markers per chromosome, since deviation of segregation ratio of *japonica* homozygote: heterozygote: *indica* homozygote from 1:2:1 suggests the linkage of the DNA probe to the mutated gene. RFLPs of 265 F₂ plants were analyzed using the land marker probes linking to this mutated gene. Distances of the mutated gene from the DNA markers were estimated by the MAPL program (Ukai 1995), which gives recombination values with the

maximum likelihood method (Allard 1956) and estimates the mapping distances by Kosambi's coefficient (Kosambi 1944).

Quantitative analysis of lysine

Meal of polished rice was suspended in 6N HCl containing 0.1% phenol in a sealed glass tube, and the protein was hydrolyzed at 105 °C for 24h. After freeze drying, the sample was suspended with 0.1N HCl, and the supernatant was subjected to amino acid analysis (Amino acid analyzer Beckman 6,300). The protein extracted from this rice meal with 0.1N NaOH was quantified by the method of Lowry *et al.* (1951).

Results and Discussion

Selection of mutants lacking α -globulin

Two mutant lines lacking α -globulin, '89WPKG30-433' and '89WPKG30-785' ('GbN-1' and 'GbN-2', hereafter), were selected from M₂ seeds of the white panicle mutant of 'Koshihikari' irradiated with gamma-rays of 300Gy. Densitometric analysis of protein bands in SDS-PAGE showed the content of α -globulin in 'Koshihikari' to be 7.5% of the total amount of the major polypeptides in the rice endosperm. The mutants lacking α -globulin had a new band of 32kDa instead (Fig. 1). The content of 32kDa polypeptide in 'GbN-1' was estimated to be 3.5% of the total amount of the major polypeptides. In the sequential extraction of globulin-albumin, prolamine, and glutelin, the 32kDa polypeptide was extracted in the glutelin fraction. Percentage of 22-23kDa glutelin basic subunits, 16kDa

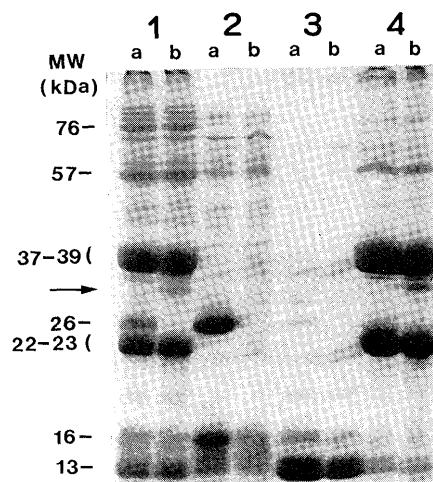


Fig. 1. SDS-PAGE analysis of globulin-albumin, prolamine, and glutelin in the seeds of an α -globulin-less mutant.

a: Koshihikari (original cultivar), b: GbN-1 (mutant)
1: total proteins, 2: globulin-albumin fraction extracted with 0.5M NaCl, 50mM KH₂PO₄-NaOH,
3: prolamine fraction extracted with 60% n-propanol, 4: glutelin fraction extracted with 1% lactic acid.

Table 1. Relative contents of major polypeptides in rice grain of an α -globulin-less mutant and the original cultivar

Molecular Weight (kDa)	76	57	37-39	32**	26*	22-23	16	13	Total (%)
Koshihikari	5.0	12.2	28.9	0	7.5	18.7	8.9	18.7	100.0
GbN-1	4.5	11.1	28.6	3.5	0	20.4	10.3	21.6	100.0

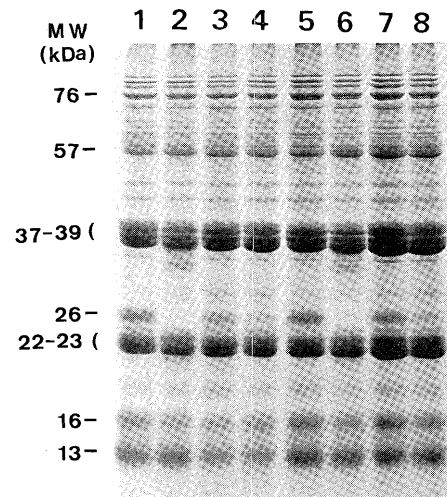
* : α -globulin, ** : a new band**Table 2.** Segregation of normal, low α -globulin, and α -globulin-less seeds in F_2 between α -globulin-less mutants and the original cultivar.

Mutant lines	Segregation of α -globulin content	Total	χ^2 (2:1:1)
	Normal:Low:Less (Expected Value)		
GbN-1	39:16:25 (40:20:20)	80	2.08 (0.3<P<0.5)
GbN-2	44:16:18 (39:19.5:19.5)	78	1.38 (0.5<P<0.7)

albumin, and 13kDa prolamine subunits increased in this mutant (Table 1).

Genetic analysis of the mutants lacking α -globulin

All selfed seeds of the plants developed from the seeds lacking α -globulin did not contain α -globulin, suggesting that these plants are homozygous for a mutated gene or genes. The F_1 seeds of the cross between the mutants and 'Koshihikari' or 'Nipponbare' had low amounts of α -globulin, when the mutants were used as a female parent. On the other hand, the F_1 seeds of the reciprocal cross between them had almost the same amount of α -globulin as 'Koshihikari' or 'Nipponbare' (Fig. 2). In the F_2 seeds between the mutants and 'Koshihikari', segregation of normal, low α -globulin, and α -globulin-less was observed. The segregation ratio of normal:low α -globulin: α -globulin-less conformed to 2:1:1, which is the result expected from the assumption that genotypes AAA and AAa of endosperm are normal, Aaa is low α -globulin, and aaa is α -globulin-less (Table 2). Therefore, the character of α -globulin-less is considered to be controlled by a single recessive gene. We tentatively named this mutated gene *glb-1(t)*. The wild type gene was partially dominant to *glb-1(t)*. The gene dose effect observed on an α -globulin content suggests that this wild type gene is the gene encoding α -globulin. All 80 F_2 seeds from the reciprocal crosses between 'GbN-1' and 'GbN-2' lacked α -globulin, indicating that the mutated genes of 'GbN-1' and 'GbN-2' are at the same locus.

**Fig. 2.** SDS-PAGE analysis of reciprocally crossed F_1 seeds between α -globulin-less mutant and normal cultivar.

1: Koshihikari (original cultivar), 2: GbN-1, 3: F_1 of Koshihikari $\times$ GbN-1, 4: F_1 of GbN-1 $\times$ Koshihikari, 5: Nipponbare (normal cultivar), 6: GbN-2, 7: F_1 of Nipponbare $\times$ GbN-2, 8: F_1 of GbN-1 $\times$ Nipponbare. The band of 26kDa is α -globulin.

Every seed lacking α -globulin had the 32kDa glutelin band and this was absent from every seed having α -globulin, suggesting that the mutation of *glb-1(t)* resulted in the synthesis of 32kDa glutelin. The gene of 32kDa glutelin would be expressed by the loss of the function of the α -globulin gene.

Expression of α -globulin gene in the mutants

In the Northern blot analysis using the α -globulin cDNA as a probe, a dense band was detected at the lane of 'Koshihikari', but no signal at the lane of α -globulin-less mutants (Fig. 3). Glutelin mRNA was detected in both 'Koshihikari' and the mutants at the same density. These results indicate that the gene of α -globulin is not expressed in these mutants, and also suggest that the mutated gene of 'GbN-1' and 'GbN-2' is the α -globulin gene itself.

Mapping of the mutated gene

In the screening of RFLP markers giving a segregation ratio deviating from 1:2:1 in 15 F_2 plants homozygous for *glb-1(t)*, *XNpb366* among 22 RFLP markers tested gave a segregation ratio of 12:3:0, suggesting that *glb-1(t)* is on chromosome 5. The *glb-1(t)* gene was mapped between *C246* and *XNpb366* and distances of *glb-1(t)* from the markers *C246* and *XNpb366* were estimated to be 15.4cM and 30.8cM, respectively (Fig. 4).

Lysine content in the mutant lacking α -globulin

Lysine content in the polished rice of the mutant line

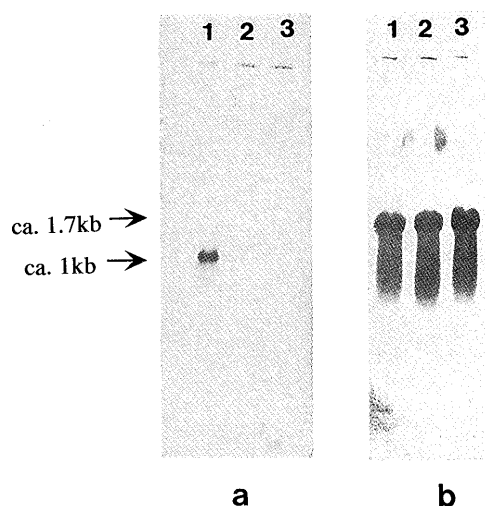


Fig. 3. Northern blot analysis of α -globulin and glutelin in developing seeds of α -globulin-less mutants.

a: α -globulin cDNA was used as a probe,
b: glutelin cDNA was used as a probe.
1: Koshihikari, 2: GbN-1, 3: GbN-2

'GbN-1' was 3.2% (molar basis) of the total amino acids, as much as that of 'Koshihikari' (3.1%). Since α -globulin occupies ca. 7.5% of the major endosperm proteins, it was expected that deletion of the protein lacking lysine would result in an 8% increase of lysine in the total amino acids. However, the lysine content of 'GbN-1' was not higher than the original cultivar. As 32kDa glutelin was synthesized instead of α -globulin in the mutant, this low value may be due to low content of lysine in 32kDa glutelin. Increase of prolamine, which contains small amount of lysine (Padhye and Salunkhe 1979), can also explain this result.

Possible use of the mutant as a breeding material

Use of only the *glb-1(t)* gene would not be effective for developing a high lysine cultivar. Shorrosh *et al.* (1992) suggested that transformation of rice with a modified α -globulin gene in which some or all of the arginine residues are replaced with lysine residues in a means of increasing lysine content in the rice endosperm. However, the expression of the modified α -globulin gene in transgenic plants may be suppressed by the intrinsic α -globulin gene, a co-suppression event. It is considered that posttranscriptional co-suppression occurs when the concentration of the same transcript rises above the threshold by transcription of an endogenous gene and a homologous transgene in addition (Matzke and Matzke 1995). A mutant in which the α -globulin gene is not expressed might be good material for the genetic engineering of rice globulin.

In the trial to obtain mutants on glutelin content, a low glutelin line 'LGC-1' was developed (Iida *et al.* 1993). 'LGC-1', which has a high prolamine content instead, is expected to be a good material for a low protein rice for the dietary cure of kidney disease because prolamine is

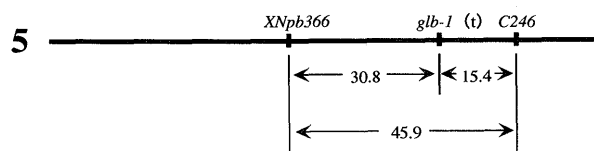


Fig. 4. Location of *glb-1(t)* in the RFLP linkage map of chromosome 5.

The genetic distances are given in Kosambi's centimorgan.

known to accumulate in the indigestible protein body, PB-I. The α -globulin and glutelin are contained in irregular-shaped protein body (PB-II) (Krishnan & White 1995), and are digestible in the human body. Combination of *glb-1(t)* and the gene for low glutelin may bring about the development of a promising low protein rice.

Acknowledgements

We are grateful to Dr. Sasaki of the Rice Genome Program in NIAR/STAFF for providing a cDNA clone of α -globulin and RFLP markers for gene mapping, to Dr. Saitou in Kyushu National Agricultural Research Station for RFLP markers, and to Dr. Takaiwa in NIAR for cDNA clones of glutelin. We also thank Prof. H. Hirano in Yokohama City University for amino acid analysis. This work was partly supported by Rice Genome Project of MAFF, Japan.

Literature Cited

- Allard, R. W. (1956) Formulas and tables to facilitate the calculation of recombination values in heredity. *Hilgardia* 24:235-278.
- Iida, S., E. Amano and T. Nishio (1993) A rice (*Oryza sativa* L.) mutant having a low content of glutelin and a high content of prolamine. *Theor. Appl. Genet.* 87:374-378.
- Juliano, B. O. (1985) Production and utilization of rice. In: *Rice: Chemistry and Technology*, 2nd ed. pp. 1-16. American Association for Cereal Chemistry, St. Paul, MN.
- Kosambi, D. D. (1944) The estimation of map distances from recombination values. *Ann Eugen* 12:172-175.
- Krishnan, H. B. and White, J. A. (1995) Morphometric analysis of rice seed protein bodies: Implication for a significant contribution of prolamine to the total protein content of rice endosperm. *Plant Physiol.* 109:1491-1495.
- Kumamaru, T., H. Satoh, N. Iwata, T. Omura, M. Ogawa and K. Tanaka (1988) Mutants for rice storage proteins. 1. Screening of mutants for rice storage proteins of protein bodies in the starchy endosperm. *Theor. Appl. Genet.* 76:11-16.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.

- Matzke, M. A. and A. J. M. Matzke (1995) How and why do plants inactivate homologous (Trans)genes? *Plant Physiol.* 107:679–685.
- Mertz, E. T., L. S. Bates and O. E. Nelson (1964) Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science* 145:279–280.
- Morita, Y. and C. Yoshida (1968) Studies on γ globulin of rice embryo. Part I. Isolation and purification of γ globulin from rice embryo. *Agr. Biol. Chem.* 32:664–670.
- Murray, M. G. and W. F. Thompson (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Res.* 8:4321–4325.
- Padhye, V. W. and D. K. Salunkhe (1979) Extraction and characterization of rice proteins. *Cereal Chem.* 56:389–393.
- Pan, S. J. and G. R. Reeck (1988) Isolation and characterization of rice α -globulin. *Cereal Chem.* 65:316–319.
- Shorrosh, B. S., L. Wen, K. C. Zen, J. K. Huang, J. S. Pan, M. A. Hermodson, K. Tanaka, S. Muthukrishnan and G. R. Reeck (1992) A novel cereal storage protein: Molecular genetics of the 19kDa globulin of rice. *Plant Mol. Biol.* 18:151–154.
- Takaiwa, F., S. Kikuchi and K. Oono (1978) A rice glutelin gene family – A major type of glutelin mRNAs can be divided into two classes. *Mol. Gen. Genet.* 208:15–22.
- Ukai, Y., R. Ohsawa, A. Saito and T. Hayashi (1995) A package of computer programs for construction of DNA polymorphism linkage maps and analysis of QTL. *Breeding Science* 4:139–142.
- Yamagata, H., T. Sugimoto, K. Tanaka and Z. Kasai (1982) Biosynthesis of storage proteins in developing rice seeds. *Plant Physiol.* 70:1094–1100.