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# Mutants having a low content of 16-kDa allergenic protein in rice (*Oryza sativa* L.)

**T. Nishio* and S. Iida**

Institute of Radiation Breeding, NIAR, MAFF, Ohmiya, Ibaraki 319-22, Japan

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**Summary.** Rice mutants containing low levels of the 16-kDa allergenic protein, which is the main allergen in the rice grain for patients of atopic dermatitis due to the intake of rice, were screened, and 4 independent mutant lines with small amounts of this protein were found by SDS gel electrophoresis and immunoblot analysis. These mutants were grouped into two types. Two mutant lines, 85KG-4 and 86RG-18, contained low levels of the 16-kDa and 26-kDa polypeptides and a high level of the 57-kDa polypeptide. The 16-kDa polypeptide content of these mutants was about half that of the original cultivars. Homozygous lines were developed, and these showed normal growth and seed set. The other 2 mutant lines, 87KG-970 and 89WPKE-149, showed traces of the 16-kDa and 26 kDa polypeptides and contained a high level of the 13 kDa polypeptide. The homozygous plants of this type were sterile. All of the mutant lines had floury endosperms. Genetic analysis suggested that low 16-kDa polypeptide content is controlled by a single recessive gene. Attempts to separate of the genes for low 16-kDa polypeptide content and floury endosperm by crossing with the original cultivar were unsuccessful, suggesting the tight linkage of these two genes or pleiotropism of a single mutated gene. The relationship between low 16-kDa polypeptide content and the floury character and the possible use of the mutant as a low allergen rice are discussed.

**Key words:** Rice – Mutation breeding – Allergenic protein – 16-kDa polypeptide – Floury

## Introduction

Many examples of atopic dermatitis resulting from rice consumption have been reported in Japan (Yamada et al. 1987). This condition occurs mainly in children, and the need for measures to cope with this situation is becoming urgent. The main allergen of rice has been identified to be the 16-kDa globulin, which is heat stable and resistant to degradation by proteolitic enzymes in the human body, as are many of the other food allergens reported so far (Matsuda et al. 1988, 1991). A method was developed to make hypoallergenic rice by digesting the allergenic protein with enzyme treatment (Watanabe et al. 1990). The hypoallergenic rice is produced commercially and supplied to patients. In this treatment, however, a large amount fo Actinase is used as a proteolitic enzyme, making the high cost of this rice unavoidable. As an alternative, highly polished rice of a cultivar having a large endosperm is also marketed as a low allergen rice, since the outer part of the endosperm has a higher protein content than the inner part. However, the inner part of rice endosperm does not have a low 16-kDa globulin content. Rice cultivars that either lack the 16-kDa globulin or have lower amounts of this protein are required as a low allergen rice.

Several mutants having an altered composition of seed proteins have been obtained by treatment with chemical mutagens (Kumamaru et al. 1988). Mutation induction and selection by SDS polyacrylamide gel electrophoreses (SDS-PAGE) is considered to be a reliable method to develop rice cultivars lacking undesirable polypeptides. In the report presented here we describe the characters of mutants having low amounts of the 16-kDa polypeptide that were obtained by gamma-ray irradiation and ethyl methansulfonat (EMS) treatment, present a genetic analysis of the mutants, and suggest the possible use of the mutant as a low allergen rice.

*Correspondence to:* T. Nishio

## Materials and methods

*Materials*

Dry seeds of rice (*Oryza sativa* cv 'Koshihikari') were either irradiated by gamma-rays from a [$^{60}$Co] source at a dose of 200 or 300 Gy at 10 Gy/h or were treated with 0.2 *M* ethyl methanesulfonate (EMS) for 5 h. The treated seeds, the $M_1$ generation, were grown in an isolated field of the Institute of Radiation Breeding, and 1,120 populations of $M_2$ seeds were produced in 1987. We also used our genetic stocks of rice mutants, 1,433 lines, which were homozygous lines having various mutated genes.

For the genetic analysis, mutants were crossed with their original cultivars, and their $F_2$ and $F_3$ seeds were subjected to protein analysis. An investigation of the components of yield of the mutant lines was carried out by growing 15 plants in a paddy field and comparing their components of yield with those of their original cultivars, 'Koshihikari' and 'Reimei'. 'Koshihikari' is a cultivar that is at present grown widely in Japan, and 'Reimei' is a cultivar that was grown widely about 20 years ago.

*Electrophoresis*

An endosperm of a mature seed was crushed with pliers and suspended in 700 µl extraction solution containing 4% sodium lauril sulfate (SDS), 8 *M* urea, 5% mercaptoethanol, 20% glycelin, and 0.125 *M* TRIS-HCl buffer (pH 6.8). After incubation at room temperature for 16 h, the homogenate was centrifuged at 10,000 *g* for 5 min, and 5 µl of the resulting supernatant was subjected to SDS-PAGE. The electrophoresis was carried out according to the method of Laemmli (1970) on a 15% acrylamide gel, and the protein was stained by Coomassie Brilliant Blue R-250. The amount of each protein was measured by a densitometer.

Salt-soluble protein (globulin and albumin) was extracted from 0.1 g of a powder of polished grains (ca. 13% weight loss from the brown rice) by gentle shaking for 16 h at 4 °C. The extracted protein was precipitated by the addition of cold acetone, dissolved in SDS-urea solution, and subjected to SDS-PAGE.

*Immunoblot analysis*

Proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride membrane by means of a Western blotting apparatus. The membrane was immersed in blocking solution, 0.5% blocking regent (Boelinger) dissolved in TRIS-buffered saline (0.15 *M* NaCl, 0.1 *M* TRIS-HCl pH 7.5), for 30 min and then incubated in TRIS-buffered saline containing rabbit polyclonal or mouse monoclonal antibody against the 16-kDa allergenic protein for 30 min. These antibodies were kindly provided by Prof. R. Nakamura of Nagoya University. After washing twice in TRIS-buffered saline for 15 min, the membrane was incubated in the same buffer containing anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) or anti-mouse IgG-alkaline phosphatase conjugate (Sigma) for 30 min. The membrane was washed twice with TRIS-buffered saline, and alkaline phosphatase activity was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

## Results and discussion

*Screening of mutants*

As a results of the screening of mutants from 1,120 populations of $M_2$ seeds and 1,433 lines in our genetic stocks, 3 independent lines displaying a thin band of the 16-kDa polypeptide (85KG-4, 86RG-18 and 87KG-970) were selected (Fig. 1 a). 85KG-4 and 86RG-18 were found in our genetic stocks of mutant lines and were mutants having a floury endosperm. 85KG-4 is a mutant line obtained by gamma-ray irradiation of the seeds of cv 'Koshihikari'. 86RG-18 was obtained from gamma-ray irradiation of a pale green mutant of cv 'Reimei'. 87KG-970 is a mutant line found by the screening of $M_2$ seeds and was derived from gamma-ray irradiation of 'Koshihikari' (Table 1).

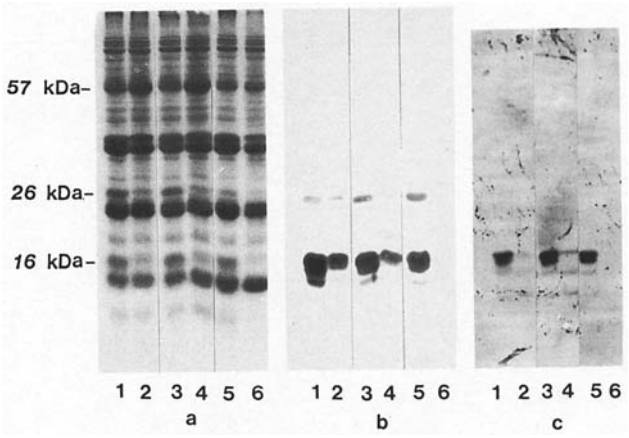


**Fig. 1 a–c.** Electrophoretic analysis of seed proteins of rice mutants. **a** Total proteins stained by Coomassie Brilliant Blue R-250, **b** detection of allergenic protein using rabbit polyclonal antibody, **c** detection of allergenic protein by mouse monoclonal antibody. *Lanes 1, 3, 5* 'Koshihikari' (normal), *lane 2* 85KG-4, *lane 4*, 86RG-18, *lane 6* 87KG-970

Immunological analysis using rabbit polyclonal antibody against the 16-kDa allergenic protein gave a dense color at the site of the 16-kDa polypeptide band of 'Koshihikari'. However, only a faint color was obtained in 85KG-4 and 86RG-18, and no color in 87KG-970 (Fig. 1 b). Analysis with mouse monoclonal antibody against the 16-kDa allergenic protein showed a color reaction at the site of the 16-kDa band of cv 'Koshihikari', while only a faint reaction was found in 85KG-4 and 86RG-18 and no reaction in 87KG-970 (Fig. 1 c).

*Characterization of mutants*

The composition of the seed proteins of these mutants was compared by quantification of the polypeptides by densitometry of SDS-PAGE stained by Coomassie Brillant Blue R-250 (Fig. 2). 85KG-4 and 86RG-18 had smaller amounts of the 16-kDa and 26-kDa polypeptides and a larger amount of 57-kDa polypeptide than 'Koshihikari'. 87KG-970 contained traces of the 16-kDa and 26-kDa polypeptides and a large amount of the 13-kDa polypeptide. A new mutant line, 89WPKE-149, which has a protein composition similar to that of 87KG-970, was found later. This mutant was obtained by EMS treatment of the seeds of a white panicle mutant of 'Koshihikari'.

**Table 1.** Mutant lines containing low levels of the 16-kDa polypeptide

| Mutant line | Original cultivar | Treatment | Protein composition | Other characters |
|---|---|---|---|---|
| 85KG-4 | Koshihikari | Gamma ray (200 Gy) | Low 16-kDa, low 26 kDa, high 57 kDa | Floury endosperm |
| 86RG-18 | Reimei Pale green leaf | Gammy ray (200 Gy) | Low 16 kDa, low 26 kDa, high 57 kDa | Floury endosperm |
| 87KG-970 | Koshihikari | Gammy ray (200 Gy) | Trace 16 kDa, trace 26 kDa, high 13 kDa | Floury endosperm, sterile |
| 89WPKE-149 | Koshihikari White panicle | EMS (0.2 *M*, 5 h) | Trace 16 kDa, trace 26 kDa, high 13 kDa | Floury endosperm, sterile |

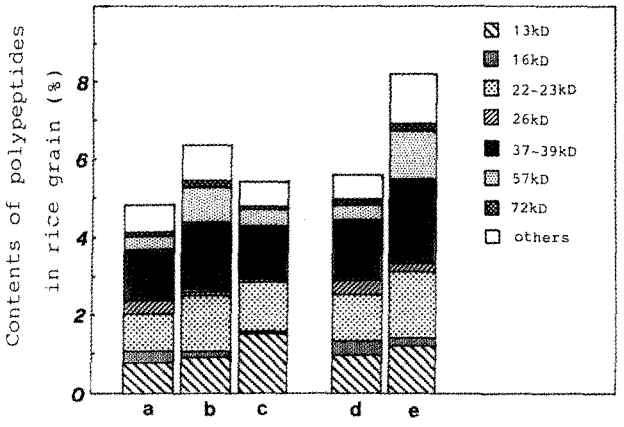


**Fig. 2.** Major polypeptide content in rice grains. *lane a* 'Koshihikari' (normal), *lane b* 85KG-4, *lane c* 87KG-970, *lane d* 'Reimei' (normal), *lane e* 86RG-18

**Table 2.** Components of yield in low allergen lines

| Characters | Koshihikari | 85KG-4 | Reimei | 86RG-18 |
|---|---|---|---|---|
| A Number of panicles in a plant | 12.0 | 11.3 | 8.1 | 8.5 |
| B Number of flowers in a panicle | 124.6 | 123.5 | 102.7 | 87.8 |
| C Number of fertile seeds in a panicle | 109.8 | 113.2 | 94.9 | 67.5* |
| D Seed fertility (%) C/B | 88.1 | 91.7 | 92.6 | 74.5** |
| E Weight of 1,000 grains (g) | 18.9 | 19.3 | 22.3 | 15.8** |
| F Percentage of grains of good quality | 96.3 | 96.4 | 94.3 | 78.9** |
| G Estimated yield per plant (g) A × C × E × F/100,000 | 24.0 | 23.8 | 16.2 | 7.2** |

* Significant at the 5% level in *t*-test between a mutant line and the original cultivar; ** significant at 1% level in t-test

Since the 16-kDa polypeptide band contains salt-insoluble polypeptides (Kumamaru et al. 1988), salt-soluble fractions were analyzed by SDS-PAGE. Though the total amount of salt-soluble proteins (albumin and globulin) in polished grains of 85KG-4 and 86RG-18 was not substantially different from that present in their original cultivars, the amount of 16-kDa protein in these mutants was less than half of that found in their original cultivars. In 87KG-970, the salt-soluble fraction was smaller than that present in the original cultivar, and the 16-kDa globulin fraction was about one-fifth the size of that of 'Koshihikari'.

Seeds of 87KG-970 and 89WPKE-149 with an altered protein composition had a floury endosperm. The plants obtained from these seeds were less vigorous and grew slowly. While these plants put forth flowers, the latter were found to be sterile. These two mutants were, therefore, maintained by selecting for heterozygotes of the mutated gene.

The agronomic characters of 85KG-4 and 86RG-18 were investigated and compared with their original cultivars (Table 2). The components of yield in 85KG-4 were not significantly different from those for 'Koshihikari', and this mutant line was expected to have the same level of yield as 'Koshihikari'. 86RG-18 showed lower values in most of the components of yield than 'Reimei' and was evaluated to have lower yield. However, the low yield of 86RG-18 may be due to its pale green character; backcrossing to 'Reimei' and the selection of normal green plants may improve the productivity of 86RG-18.

Both 85KG-4 and 86RG-18 had floury endosperms, but the outer parts of their endosperm were normal while the inner parts were floury. Although floury seeds tend to be broken by the polishing process, brown rice of 85KG-4 could be processed to white rice by repeated polishing under mild conditions. The taste of the cooked rice of 85KG-4 was not substantially inferior to that of the original cultivar, 'Koshihikari', a leading Japanese cultivar that has the highest cooking quality.

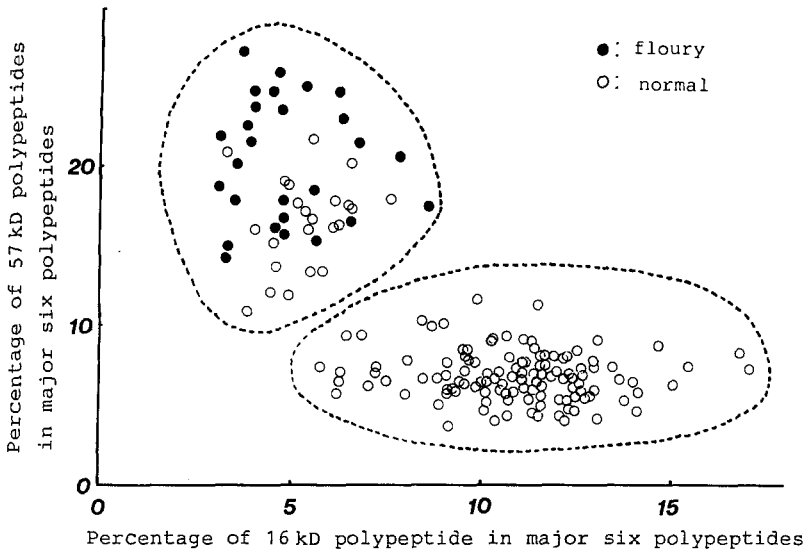


**Fig. 3.** Amounts of 16-kDa and 57-kDa polypeptides in seeds of $F_2$ between 'Koshihikari' and 85KG-4

**Table 3.** Segregation of the low 16-kDa trait and the floury character in the $F_2$ between 85KG4 and 'Koshihikari'

| | Normal | Low 16 kDa | Total |
|---|---|---|---|
| Normal | 138 | 23 | 161 [b] |
| Floury | 0 | 27 | 27 |
| Total | 138 [a] | 50 | 188 |

[a] $\chi^2$ (3:1) = 0.26
[b] $\chi^2$ (3:1) = 11.3 ($P < 0.01$)

*Genetic analysis*

The inheritance of an altered protein composition and floury endosperm in these mutants was investigated using $F_2$ seeds. In the cross between 85KG-4 and 'Koshihikari', all $F_2$ seeds having a reduced 16-kDa allergenic protein trait contained low amounts of the 26-kDa polypeptide and high amounts of the 57-kDa polypeptide, suggesting that these three characters of 85KG-4 are controlled by the same mutated gene or closely linked genes (Fig. 3). Segregation of these mutant characters in the $F_2$ population corresponded almost exactly to the ratio of 3:1, revealing the genetic control of this mutation by a single recessive gene (Table 3).

All of the floury grains contained smaller amounts of the 16-kDa polypeptide, though some grains containing lower amounts of the 16-kDa polypeptide were not floury. Segregation of the floury character in the $F_2$ deviated from the 3:1 ratio. One-half of a grain was subjected to protein analysis, and the other half grain bearing the embryo was sown on a moist filter paper to grow a plant. The visually normal seed (non-floury) which contained a lower amount of the 16-kDa polypeptide gave floury seeds in the self-pollinated progenies. The most probable explanation is that the floury character, being controlled by a single recessive gene, is influenced by physiological and environmental conditions. Some seeds of 85KG-4 were evidently normal, although 85KG-4 was an inbred line maintained by repeated selfing. Our trial to separate the genes controlling smaller amounts of the 16-kDa polypeptide and the floury character in 85KG-4 was unsuccessful, suggesting the tight linkage of the mutated genes of these characters or pleiotropism of a single mutated gene.

A similar result was obtained in the segregation in the $F_2$ of 86RG-18 and its original line. Whether the mutated gene of 86RG-18 is on the same locus as that of 85KG-4 has not yet been tested.

In 87KG-970 and 89WPKE-149 maintained by selfing of a heterozygote, a reduction in the level of the 16-kDa polypeptide was always accompanied by a decrease in the level of the 26-kDa polypeptide and an increase in the level of the 13-kDa polypeptide. About one-forth of the seeds had undetectable amounts of 16-kDa and 26-kDa polypeptide and a high content of 13-kDa polypeptide, suggesting the control of these characters by a single recessive gene. All of the seeds having these characters were floury without exception.

*Use of the mutants as a low allergen rice*

The floury character of 85KG-4 was limited only in the inner part of the endosperm, and the grains could be polished for cooking rice. The cooking quality of this mutant was not substantially inferior to that of the original cultivar, 'Koshihikari'. The estimated yield of 85KG-4 was almost the same as that of 'Koshihikari'.

The 16-kDa polypeptide content of this mutant was about half that of the original cultivar. Whether rice containing a small amount of the 16-kDa allergenic

protein is clinically valuable still remains to be examined. Even if it is difficult to use this mutant directly as a low allergen rice, it could be good material for the production of hypoallergenic rice by enzyme treatment. In our preliminary investigation, it was possible to save on Actinase by using this mutant as a material for producing rice having a protein composition similar to that of the marketed hypoallergenic rice.

Two mutant lines, 87KG-970 and 89WPKE-149, had trace amounts of the 16-kDa polypeptide and therefore were expected to be useful as a low allergen rice. Although homozygous plants of these mutated genes were sterile, heterozygotes can be maintained and propagated in a greenhouse. Since the homozygotes have floury endosperm, they can be selected for by means of a sorting apparatus. The grains can be used for rice meal.

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