Mutants for rice storage proteins.

IV. Maternally inherited mutants for storage proteins of protein bodies in the starchy endosperm

Toshihiro Kumamaru,‡
Hikaru Satoh,*
Takeshi Omura and
Masahiro Ogawa†

Plant Breeding Laboratory, Faculty of Agriculture,
Kyushu University, Hakozaki, Fukuoka 812, Japan.

* Institute of Genetic Resources, Faculty of
Agriculture, Kyushu University, Hakozaki, Fukuoka
812, Japan.

† The Research Institute for Food Science, Kyoto
University, Uji, Kyoto 611, Japan.

Maternal inheritance, a phenomenon extremely rare in plants, was observed in two mutants for rice storage protein in the starchy endosperm. When these mutants, 10/13a-L and 10/16-H, were crossed reciprocally with their original variety, Kinmaze, the F_1 seeds in all crosses showed the same character as the maternal parent. The F_2 seeds of the reciprocal crosses between Kinmaze and 10/13a-L were also of normal type, but those between Kinmaze and 10/16-H showed 10/16-H type. In 10/13a-L, the segregation ratio of F_2 plants observed in F_3 seeds showed a good fit to the expected 3:1 ratio, and in 10/16-H, the segregation ratio of F_2 plants observed in F_3 seeds showed a good fit to the expected 1:3 ratio. From the results of F_1 , F_2 and F_3 seeds, it was concluded that the mutants were expressed according to the genetic behaviour of maternal inheritance governed by plant genotype, and that 10/13a-L and 10/16-H were controlled by a single recessive and dominant gene, respectively. SDS-PAGE analysis indicated that both mutant genes were regulatory genes for the storage proteins. Based on the results of allelism tests, the genes for 10/13a-L and 10/16-H were designated as *esp-3* and *Esp-4*, respectively. Since *esp-1*, the gene controlling the 13b-L, a mutant of rice storage protein, produces loss of the effect of maternal inheritance of *esp-3*, it is assumed that *esp-1* is epistatic to *esp-3*.

INTRODUCTION

The improvement of rice storage proteins is important for the Asian diet. Studies on rice, however, have generally been limited to ways of increasing the total content of storage proteins (Beachell *et al.*, 1972; Higashi *et al.*, 1974; Kambayashi *et al.*, 1984; Osone and Takagi, 1970) or increasing the lysine content in the storage proteins (Shin *et al.*, 1977). However, due to the fact that the gene(s) for rice storage proteins themselves has never been identified, the qualitative improvement of the rice proteins themselves remains virtually unexplored.

Rice storage proteins are accumulated in two types of protein bodies in the starchy endosperm (Tanaka *et al.*, 1980). Rice prolamin is stored in protein body type I (PB-I), which are spherical proteinaceous particles of lamellar structure, 1–

2 μm in diameter. Rice glutelin is deposited in protein body type II (PB-II), a uniformly electron dense structure, 2–3 μm in diameter. In SDS-PAGE analysis, PB-I shows 10 kDa, 13a, 13b and 16 kDa polypeptides; and PB-II shows 22–23 kDa, 26 kDa and 37–39 kDa polypeptides (Ogawa *et al.*, 1987; Tanaka *et al.*, 1980; Yamagata *et al.*, 1982).

Since PB-I is hard to digest by humans, whereas PB-II is easily digestible (Ogawa *et al.*, 1987; Tanaka *et al.*, 1975a,b), PB-I is regarded as nutritionally inferior to PB-II. Hence, to improve the nutritional value of the endosperm storage proteins, it is desirable either to increase PB-II content and/or to decrease PB-I.

In a previous paper, we reported four types of mutants for rice storage protein showing (1) 13b polypeptide low; (2) 10 kDa and 16 kDa polypeptide high and 13b polypeptide low; (3) 10 kDa and 13a polypeptide low; (4) 57 kDa polypeptide high. These mutants were named 13b-L, 10/16-H, 10/13a-L and 57-H, respectively. Mutant lines CM

‡ Present address: Plant Breeding and Genetics Research Laboratory, Japan Tobacco Inc., Higashibara, Toyoda, Iwata, Shizuoka 438, Japan.

21 and CM 1834, which were 13b-L and 10/16-H, respectively, had an increased glutelin content (Kumamaru *et al.*, 1988). It has been shown that 13b-L in CM 21, 10/16-H in CM 1834 and 10/13a-L in mutant line CM 1675 are the mutants for the polypeptides in PB-I (Ogawa *et al.*, 1989). To evaluate their genetic potential for the improvement of rice storage protein, it is necessary to determine the genetic behaviour of these mutant polypeptides. Thus far, experiments done on 13b-L in CM 21 and 57-H in CM 1787 have demonstrated that each of the mutants was controlled by a single recessive gene, which was a mutant of the regulatory gene for the polypeptides in the protein body, and these genes for 13b-L and 57-H were designated as *esp* (endosperm storage protein)-1 and *esp*-2, respectively (Kumamaru *et al.*, 1987). The present study further deals with the genetic behaviour of the mutant genes for the polypeptides in CM 1675 and CM 1834.

MATERIALS AND METHODS

Materials

Four mutant lines for seed storage protein, CM 21, CM 1787, CM 1675 and CM 1834, were used in these experiments. They were induced by *N*-methyl-*N*-nitrosourea (MNU) from rice cultivar, Kinmaze, as reported elsewhere (Sato and Omura, 1979), and have been maintained at the Plant Breeding Laboratory, Kyushu University. Fig. 1 and fig. 3 show the SDS-PAGE of the proteins in CM 1675 and CM 1834, respectively, which deal with the genetic behaviour in this work, and Kinmaze. In Kinmaze, the intensity of 13a (a larger component of 13 kDa) and 13b (a smaller component of 13 kDa) were almost equal. But the intensity of the 13a polypeptide band was lower than that of the 13b polypeptide band in CM 1675, while the pattern of intensities was reversed in CM 1834. In CM 1675, the intensity of the 10 kDa polypeptide band was lower than that of Kinmaze. In CM 1834, the intensity of the 10 and 16 kDa polypeptide bands was higher than those of Kinmaze. So, the mutant type in CM 1675 and CM 1834 was named 10/13a-L and 10/16-H, respectively (Kumamaru *et al.*, 1988).

Crosses

To investigate the genetic behaviour of the mutant polypeptides in CM 1675 and CM 1834, reciprocal crosses were made between CM 1675 and CM 1834, and Kinmaze. The proteins of F_1 , F_2 and F_3

seeds of each cross were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To test allelism, crosses between mutant lines were made and F_1 , F_2 and F_3 seeds were subjected to SDS-PAGE analysis.

Protein extraction

Individual rice seed was crushed with pliers, and the tissue was placed in a micro test tube (1.5 ml) with 700 μ l of 0.125 M Tris-HCl pH 6.8 containing 4 per cent SDS, 6 M urea, 5 per cent 2-mercaptoethanol and 20 per cent glycerin and incubated at 30°C for 4 hours. After centrifugation (5 min, 15,000 r.p.m.), the supernatants were applied to SDS-PAGE.

SDS-PAGE

Extracted storage proteins of rice seeds were electrophoresed using the discontinuous buffer system of Laemmli (1970) on a slab gel, and were stained by 0.05 per cent Coomassie brilliant blue R-250 in 7 per cent acetic acid and 50 per cent methanol, and destained by 7 per cent acetic acid and 25 per cent methanol.

RESULTS AND DISCUSSION

Fig. 1 shows the SDS-PAGE of the proteins of F_1 and F_2 seeds from the reciprocal crosses between Kinmaze and CM 1675. In the cross between Kinmaze (φ) and CM 1675 (δ), the SDS-PAGE pattern of F_1 seed proteins showed the normal type, i.e., the Kinmaze type (F_1 -1). In the cross between CM 1675 (φ) and Kinmaze (δ), the SDS-PAGE pattern of F_1 seed proteins showed the 10/13a-L type, i.e., the CM 1675 type (F_1 -2). The SDS-PAGE pattern of F_2 seed proteins obtained from the reciprocal crosses showed the normal type. From the expression of the character of seed parents in F_1 seeds, it is assumed that the expression of 10/13a-L is dependent on either the genotype of the plant, or the cytoplasm. The results of F_2 seeds indicate that the expression of the mutant is dependent on the former. This led us to speculate that the genotype of the F_1 plant is heterozygous for 10/13a-L and, from the results of F_2 seeds, that 10/13a-L is recessive to the normal type.

In order to confirm the above supposition, F_2 plants were cultivated, and the protein of a group of single F_3 seed per F_2 plant was analyzed by SDS-PAGE, the results of which are shown in fig.

Figure 1 SDS-PAGE analysis of the storage protein in F_1 and F_2 seeds of the cross between Kinmaze and CM 1675. (F_1 -1) F_1 seed of the cross between Kinmaze (♀) and CM 1675 (♂). (F_1 -2) F_1 seed of the cross between CM 1675 (♀) and Kinmaze (♂).

2. Normal and 10/13a-L types were segregated in the F_3 seeds. Moreover, in the F_3 seeds, there was no segregation of individual F_2 plants (data not shown). These findings suggested that 10/13a-L in CM 1675 is determined by the genotype of the plant, not the endosperm. In the cross between CM 1675 and Kinmaze, the segregation mode in

F_2 plants investigated by F_3 seeds are shown in table 1. The segregation ratio of normal and 10/13a-L types showed a good fit to the expected 3:1 ratio. These results suggest that 10/13a-L in CM 1675 is controlled by a single recessive gene.

Fig. 3 shows the SDS-PAGE of the proteins of F_1 and F_2 seeds from the reciprocal crosses between

Figure 2 SDS-PAGE analysis of the storage protein in a group of single F_3 seed per F_2 plant of the cross between Kinmaze and CM 1675.

Table 1 The phenotype of F₁ and F₂ seeds, and segregation of normal and 10/13a-L types in F₂ plants as seen in F₃ seeds of the reciprocal cross between Kinmaze and CM 1675

Cross combination		Phenotype of F ₁ seeds	Phenotype of F ₂ seeds	Segregation in F ₂ plants			χ^2 (3:1)
♀	♂			Normal	10/13a-L	Total	
Kinmaze × CM 1675		Normal	Normal	118	43	161	0.25
CM 1675 × Kinmaze		10/13a-L	Normal	—	—	—	—

— = No data.

Kinmaze and CM 1834. In the cross between Kinmaze (♀) and CM 1834 (♂), F₁ seed showed the normal type (F₁-2), and in the cross between CM 1834 (♀) and Kinmaze (♂), F₁ seed showed the 10/16-H type, *i.e.*, the CM 1834 type (F₁-2). All F₂ seeds of the reciprocal crosses showed the 10/16-H type. If the 10/16-H in CM 1834 is determined by the genotype of the plant, such as 10/13a-L in CM 1675, then, judging from the results of F₂ seeds, it would seem that the 10/16-H is dominant to the normal type.

Fig. 4 shows the SDS-PAGE of the proteins of F₃ seeds from the cross between Kinmaze and CM 1834. Normal and 10/16-H types were segregated in the F₃ seeds. Since there was no segregation in the F₃ seeds of individual F₂ plants (data not shown), it is thought that 10/16-H in CM 1834 is also determined by the genotype of the plant. The segregation mode in F₂ plants investigated by F₃ seeds from this cross is shown in table 2. The

segregation ratio of normal and 10/16-H types showed a good fit to the expected 1:3 ratio. The results suggest that 10/16-H in CM 1834 is controlled by a single dominant gene.

The above genetic phenomenon, in which the character of the endosperm is controlled by plant genotype, is known as "maternal inheritance". This phenomenon, which is extremely rare in plants, has been observed in a few cases such as the egg colour (Uda, 1923) and maternal chocolate mutant (Yoshimura, 1981; 1985) in silkworm.

Gene dosage effect has been reported to occur in F₁ and F₂ seed storage protein in barley (Doll, 1980), wheat (Burnouf *et al.*, 1983; Dhaliwal, 1977) and maize (Soave *et al.*, 1981; Valentini *et al.*, 1979). In the case of mutants for the structural genes encoding polypeptides, the gene dosage effect, if any, should appear in the polypeptides of F₁ and F₂ seeds. *esp-1* and *esp-2* which are genes for 13b-L in CM 21 and 57-H in CM 1787, respec-

Figure 3 SDS-PAGE analysis of the storage protein in F₁ and F₂ seeds of the cross between Kinmaze and CM 1834. (F₁-1) F₁ seed of the cross between Kinmaze (♀) and CM 1675 (♂). (F₁-2) F₁ seed of the cross between CM 1675 (♀) and Kinmaze (♂).

Figure 4 SDS-PAGE analysis of the storage protein in a group of single F_3 seed per F_2 plant of the cross between Kinmaze and CM 1834.

tively, are regarded as mutants for regulatory genes of the polypeptides, for they showed no gene dosage effect of this type (Kumamaru *et al.*, 1987). Since the genes for 10/13a-L and 10/16-H also revealed no such gene dosage effect, and since the mutated polypeptides were inherited together (figs. 2 and 4), it is likely that the genes are mutations of the regulatory gene for the polypeptides, and are analogous to the cases of *esp-1* and *esp-2*.

To test allelism in the genes for 10/13a-L and 10/16-H, reciprocal crosses were made of CM 1675 and CM 1834 with CM 21 (*esp-1*) and CM 1787 (*esp-2*). The results of SDS-PAGE analysis of F_1 seeds are shown in table 3. In cases where CM 21 and CM 1787 were used as the seed parent, all seeds indicated normal type. This suggests that the genes for both 10/13a-L and 10/16-H are located at different locus from *esp-1* and *esp-2*. In order

Table 2 The phenotype of F_1 and F_2 seeds, and segregation of normal and 10/16-H types in F_2 plants as seen in F_3 seeds of the reciprocal cross between Kinmaze and CM 1834

Cross combination		Phenotype of F_1 seeds	Phenotype of F_2 seeds	Segregation in F_2 Plants			χ^2 (1:3)
♀	♂			Normal	10/16-H	Total	
Kinmaze	CM 1834	Normal	10/16-H	39	120	159	0.02
CM 1834	Kinmaze	10/16-H	10/16-H	—	—	—	—

— = No data

Table 3 The phenotype of F_1 seeds of reciprocal crosses among 4 mutant lines

♀ \ ♂	CM 21	CM 1787	CM 1675	CM 1834
CM 21 (13b-L)	—	—	+	+
CM 1787 (57-H)	—	—	+	+
CM 1675 (10/13a-L)	+	10/13a-L	—	—
CM 1834 (10/16-H)	10/16-H	10/16-H	10/16-H	—

+ = Normal type, — = No data.

Table 4 Segregation of normal and mutant types in F₂ and F₃ seeds of the crosses between CM 1675 and two mutant lines

Cross combination	Segregation in F ₂ seeds			$\chi^2(3:1)$	Segregated types in F ₃ seeds
	Normal	Mutant	Total		
CM 1675 × CM 21	35	11*	46	0.57	Normal, 13b-L, 10/13a-L
× CM 1787	42	8**	50	2.16	Normal, 57-H, 10/13a-L and 57-H

* 13b-L type (*esp-1*), ** 57-H type (*esp-2*)

to confirm the results obtained by F₁ seeds, F₂ and F₃ seeds of the combination were analyzed.

The segregation mode in F₂ and the segregated types in F₃ seeds from the cross between CM 1675 and two mutant lines are shown in table 4. In F₂ seeds, the segregation ratio of normal types, and *esp-1* and *esp-2*, respectively, showed a good fit to the expected 3:1 ratio. In F₃ seeds of the two combinations, the parent types of the combination and normal type segregated. The results obtained by F₂ and F₃ seeds lend support to the argument that the gene for 10/13a-L in CM 1675 is located at a different locus from *esp-1* and *esp-2*, hence the gene was designated as *esp-3*.

The segregation mode in F₂ seeds and segregated types in F₃ seeds from the crosses between CM 1834 and three mutant lines are shown in table 5. F₂ seeds obtained from crosses between CM 1834 and CM 21 and CM 1675, respectively, indicated only the 10/16-H type, while in the cross combination of CM 1834 and CM 1787, the segregation of 10/16-H and 10/16/57-H types showed a good fit to the expected 3:1 ratio. In F₃ seeds from all combinations, the parent types of the combination and normal type segregated. The results obtained by F₂ and F₃ seeds support the fact that the gene for 10/16-H is located at a different locus from *esp-1* and *esp-2*. They further indicate that this gene is located at a different locus from *esp-3*, hence, the gene for 10/16-H in CM 1834 was designated as *Esp-4*.

In table 3, where CM 21 was crossed with the pollen parent, F₁ seeds of the combinations with CM 1675 indicated normal type, hence, it is assumed that *esp-1* is epistatic to the *esp-3*. This

point, however, could not be confirmed in the F₂ and F₃ seeds of this study, and remains the subject of future study.

Yamagata and Tanaka (1986) reported that the protein accumulated in PB-I appears to be synthesized by membrane bound polysomes attached to PB-I or the rough endoplasmic reticulum, before they pass through the membrane into the lumen where they are deposited in aggregate form. Of *esp-1*, *esp-3* and *Esp-4*, which are mutants for PB-I (Kumamaru *et al.*, 1988; Ogawa *et al.*, 1989), *esp-1* is controlled by the seed genotype (Kumamaru *et al.*, 1987). By contrast, the present study demonstrated that *esp-3* and *Esp-4* are controlled by the plant genotype. The mechanism governing expression in maternally inherited mutants remains unclear, but the following two points may be tentatively proposed. First, the part of the organelle involved in the synthesis of the storage proteins, such as the membrane, is transmitted from the embryo sac to the endosperm, but degenerates in the embryo. Second, the storage proteins, or their precursors, are synthesized in the plant, shifted into endosperm and accumulated in PB-I. Furthermore, the fact that the proteins which accumulate in PB-I are governed by seed and plant genotypes implies that the system of the genetic control for protein accumulation in PB-I is extremely complex. It is expected that the mechanism of the protein accumulation into PB-I will be elucidated by utilization of the *esp-1*, *esp-3* and *Esp-4* mutants.

It is essential to make PB-II increase and/or PB-I decrease for the improvement of the nutritional value of the endosperm. It has been shown

Table 5 Segregation of normal and mutant types in F₂ and F₃ seeds of the crosses between CM 1834 and three mutant lines

Cross combination	Segregation in F ₂ seeds			$\chi^2(3:1)$	Segregated types in F ₃ seeds
	10/16-H	Double	Total		
CM 1834 × CM 21	40	0	40	—	Normal, 13b-L, 10/16-H
× CM 1787	29	11*	40	0.13	Normal, 57-H, 10/16-H, 10/16/57-H
× CM 1675	40	0	40	—	Normal, 10/13a-L, 10/16-H

* 10/16/57-H type.

that 10/13a-L, which is controlled by *esp-3*, is a mutant affecting PB-I decrease, and that 10/16-H, which is controlled by *Esp-4*, is a mutant causing higher glutelin accumulation in PB-II than in Kinmaze (Kumamaru *et al.*, 1988). However, whether the increase of glutelin is due to the pleiotropism of *Esp-4* or to the action of other genes, remains the subject of future study. These mutant lines, once utilized, will no doubt enable qualitative improvement of rice storage proteins.

REFERENCES

- BEACHELL, H. M., KHUSH, G. S. AND JULIANO, B. O. 1972. Breeding for high protein content in rice. In *Rice Breeding*, Int. Rice Res. Inst., Los Baños, Philippines, pp 419-428.
- BURNOUF, T., BOURIQUET, R. AND POUILLARD, P. 1983. Inheritance of glutenin subunits in F_1 seeds of reciprocal crosses between european hexaploid wheat cultivars. *Theor. Appl. Genet.*, **64**, 103-107.
- DHALIWAL, H. S. 1977. Genetic control of seed proteins in wheat. *Theor. Appl. Genet.*, **50**, 235-239.
- DOLL, H. 1980. A nearly non-functional mutant allele of the storage protein locus *Hor2* in barley. *Heredity*, **93**, 217-222.
- HIGASHI, T., KUSHIBUCHI, K. AND ITO, R. 1974. Studies on breeding for high protein rice. I. Protein content of different rice varieties and their relations with some agronomic traits including yield (in Japanese with English summary). *Jpn. J. Breed.*, **24**, 88-96.
- KAMBAYASHI, M., TSURUMI, I. AND SASAHARA, T. 1984. Genetic studies on improvement of protein content in rice grain (in Japanese with English summary). *Jpn. J. Breed.*, **34**, 356-363.
- KUMAMARU, T., SATOH, H., IWATA, N., OMURA, T. AND OGAWA, M. 1987. Mutants for rice storage proteins. III. Genetic analysis of mutants for storage proteins of protein bodies in the starchy endosperm. *Jpn. J. Genet.*, **62**, 333-339.
- KUMAMARU, T., SATOH, H., IWATA, N., OMURA, T., OGAWA, M. AND TANAKA, K. 1988. Mutants for rice storage proteins. I. 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.
- OGAWA, M., KUMAMARU, T., SATOH, H., IWATA, N., OMURA, T., KASAI, Z. AND TANAKA, K. 1987. Purification of protein body-I of rice seed and its polypeptide composition. *Plant Cell Physiol.*, **28**, 1517-1527.
- OGAWA, M., KUMAMARU, T., SATOH, H., OMURA, T., PARK, T., SHINTAKU, K. AND BABA, K. 1989. Mutants for rice storage proteins. 2. Isolation and characterization of protein bodies from rice mutants. *Theor. Appl. Genet.*, **78**, 305-310.
- OSONE, K. AND TAKAGI, T. 1970. Studies on breeding for high protein content and quality in rice. I. Estimation of seed-protein content using the dye-binding method. *Jpn. J. Breed.*, **20**, 301-304.
- SATOH, H. AND OMURA, T. 1979. Induction of mutation by the treatment of fertilized egg cell with N-methyl-N-nitrosourea in rice. *J. Fac. Agr., Kyushu Univ.*, **24**, 165-174.
- SHIN, Y. B., TANAKA, S. AND KATAYAMA, T. 1977. Studies on the quantitative and qualitative improvement of rice. Application of DBC as a screening technique and assessment of Japanese local rice varieties for high protein and lysine (in Japanese with English summary). *Sci. Bull. Fac. Agr., Kyushu Univ.*, **31**, 145-150.
- SOAVE, C., REGGIANI, R., FONZO, N. D. AND SALAMINI, F. 1981. Clustering of genes for 20 kd zein subunits in the short arm of maize chromosome 7. *Genetics*, **97**, 363-377.
- TANAKA, K., SUGIMOTO, T., OGAWA, M. AND KASAI, Z. 1980. Isolation and characterization of two types of protein bodies in the rice endosperm. *Agric. Biol. Chem.*, **44**, 1633-1639.
- TANAKA, Y., HAYASHIDA, S. AND HONGO, M. 1975a. Quantitative relation between feces protein particles and rice protein bodies (in Japanese with English summary). *J. Agric. Chem. Soc. Jpn.*, **49**, 425-429.
- TANAKA, Y., HAYASHIDA, S. AND HONGO, M. 1975b. The relationship of the feces protein particles to rice protein bodies. *Agric. Biol. Chem.*, **39**, 515-518.
- UDA, H. 1923. On "maternal inheritance". *Genetics*, **8**, 322-335.
- VALENTINI, G., SOAVE, C. AND OTTAVIANO, E. 1979. Chromosomal location of zein genes in *Zea mays*. *Heredity*, **42**, 33-40.
- YAMAGATA, H., SUGIMOTO, T., TANAKA, K. AND KASAI, Z. 1982. Biosynthesis of storage proteins in developing rice seeds. *Plant Physiol.*, **70**, 1094-1100.
- YAMAGATA, H. AND TANAKA, K. 1986. The site of synthesis and accumulation of rice storage proteins. *Plant Cell Physiol.*, **27**, 135-145.
- YOSHIMURA, Y. 1981. Maternally conditioned mutant for the body color of newly hatched larva in the silkworm (in Japanese). *J. Seric. Sci. Jpn.*, **50**, 206-212.
- YOSHIMURA, Y. 1985. Linkage studies on the "maternal chocolate" mutant in the silkworm, *Bombyx mori* (in Japanese). *J. Seric. Sci. Jpn.*, **54**, 208-212.