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Mutants for rice storage proteins

2. Isolation and characterization of protein bodies from rice mutants

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Summary. Rice storage proteins of the endosperm are localized in two types of protein bodies, PB-I and PB-II. Protein bodies were isolated by sucrose density gradient centrifugation from developing endosperm of three rice mutants, *CM 21, CM 1675* and *CM 1834,* and characterized after pepsin-digestion treatment by protein contents determination. Mutant protein bodies (PBs) except for their internal structure, were similar in shape and density to PB-I of the variety Kinmaze. Electrophoretic analysis of PB-I polypeptides revealed that SDS (Sodium dodecylsulfate) bands of 13 and 16 kilodaltons consisted, respectively, of four and two individual polypeptides with different pI values, while the 10-kilodalton band behaved as a single polypeptide after isoelectric focussing (IEF) electrophoresis. The differences in the polypeptide composition induced by mutants were due to the decrease and/or increase in the content of specific PB-I polypeptides. Electron microscopic observations revealed that the typical lamellar structure of the PB-I is not visible in *CM 1675*. On the contrary, the inner portion of PB-I in *CM 1834* and *CM 21* showed higher electron density than that of the variety Kinmaze. On these two mutants, the content of pepsin-indigestible and -digestible proteins were similar to those of Kinmaze, although the values of the PB-II/PB-I ratio were greater than those for Kinmaze, suggesting that these two mutants are high-glutelin rice mutants.

Key words: Endosperm – Mutant – *Oryza sativa* L. – Protein body – Storage protein

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Introduction

Rice storage proteins in the starchy endosperm are localized in two types of protein bodies called PB-I and PB-II (Tanaka et al. 1980). The major rice storage protein, glutelin, consists of groups of 20- and 40-kilodalton polypeptides and is deposited in PB-II (Tanaka et al. 1980; Zhao et al. 1983; Wen and Luthe 1985; Krishnan and Okita 1986; Krishman et al. 1986; Tanaka and Ogawa 1986). The proteins present in PB-I belong to the 13-kilodalton polypeptide group, and are prolamin in nature (Tanaka et al. 1980; Ogawa et al. 1987). In addition, PB-I contains polypeptides with apparent molecular masses (MMr) of 10 and 16 kilodaltons. Proteins present in PB-I and PB-II account, respectively, for 20% and 60% of the total proteins of rice starchy endosperm (Ogawa et al. 1987).

Because of its high lysine content, the nutritional value of rice protein is superior to other cereal proteins such as those of wheat, barley and maize (Juliano 1985). Rice grains, however, contain indigestible fecal protein particles (FPP) (Tanaka et al. 1975a, b). Recently, Ogawa et al. (1987) found that FPPs are actually PB-I. If the digestibility of PB-I proteins can be improved, this would increase the overall nutritional value of rice protein. Hibino et al. (1989) purified the 10-, 13- and 16-kilodalton polypeptides and determined their amino acid compositions. They also found that the 13-kilodalton prolamins consisted of no less than three polypeptides with different pI values, and that one of them contained more lysine than the other two. According to their data, the sulfur-

containing amino acid contents of the 10- and 16-kilodalton polypeptides were higher than that of other rice proteins.

We have screened rice mutants induced by the treatment of fertilized egg cell with N-methyl-N-nitrosourea, focussing on variants affecting protein compositions of PB-I and PB-II. Four mutant lines – *CM 21, CM 1675, CM 1834* and *CM 1787* – indicated also as *13b-L, 10/13a-L, 10/16-H* and *57-H,* were isolated (Kumamaru et al. 1988). In the *13b-L* mutation, the contents of the 13b polypeptide was relatively low, while *10/13a-L* had lower amounts of 10 kilodaltons and 13a polypeptides. In the *10/16-H* mutation, the contents of both the 10- and 16-kilodalton polypeptides increased, while 13b decreased. The *57-H* mutation showed a higher content of 57-kilodalton polypeptide and lower amounts of glutelin, compared to Kinmaze. The *13b-L* and *57-H* mutations are controlled by single recessive genes (Kumamaru et al. 1987), while *10/13a-L* and *10/16-H* mutations show maternal inheritances (Kumamaru et al. 1989). Unlike the case of *CM 1787,* which affects PB-II, *CM 21, CM 1675* and *CM 1834* possibly induce modifications of the polypeptides of PB-I.

The present study considered the purification and characterization of PB-I from the mutants *CM 21, CM 1675* and *CM 1834*. The specific proteins in PB-I and PB-II in each mutant were also determined.

Materials and methods

Plant materials

Rice mutants were obtained as described by Satoh and Omura (1979, 1981). Mutants *CM 21, CM 1675,* and *CM 1834* were grown in water culture (Yoshida et al. 1976) and 15 to 20-day-old developing grains were used for the isolation of protein bodies.

PB isolation. PBs were isolated by sucrose density gradient centrifugation and purified by pepsin digestion treatment (Ogawa et al. 1987).

Electrophoretic analysis of proteins in purified PBs. Purified PBs were suspended in IEF solution (O'Farrell 1975), sonicated for several minutes to denature proteins and centrifuged at 15,000 rpm for 15 min. The supernatant was subjected to IEF electrophoresis, and the resultant IEF gel was loaded on to a 14% polyacrylamide gel and electrophoresed, as described elsewhere (Kumamaru et al. 1988).

Electron microscopic observation. Isolated PBs were fixed with 0.2% glutaraldehyde and 4% paraformaldehyde in 20 m*M* piperazine-N,N-bis (2-ethane sulfonic acid) (PIPES) (pH 7.0) for 2 h on ice, followed by postfixing with 1% osmium tetroxide for 1 h on ice. The fixed samples were rinsed with the same buffer and dehydrated with ethanol solution. These samples were then embedded in Spurr's low viscosity resin after imbibition in a mixture of resin and acetone overnight. Thin sections (0.1 μm) were prepared with an ultramicrotome (Reichert) and the sections were stained with uranylacetate and lead citrate solutions. Electron microscopical observation was carried out with a transmission electron microscope (JEM 200C, Japan Electron Optics Lab, Tokyo).

Protein analysis of PB-I and PB-II by pepsin-digestion treatment. Fifty milligrams of powdered rice was suspended in 1 ml of 0.5 *M* NaCl in 50 m*M* TRIS-HCl (pH 7.5) solution for extraction of salt soluble proteins. The extraction procedure was repeated three times. The extracted solutions were used for the determination of salt-soluble protein content. The residue was suspended in 1 ml of pepsin buffer (0.2 *M* sodium acetate-HCl buffer pH 1.7) and 10 μl of 1% pepsin solution (3,200–3,800 units/mg, Sigma Chemical Co., St. Louis/MO and incubated for 1 h at 37 °C. After centrifugation, the resultant pellets were used for determination of pepsin-indigestible protein content. After washing the pellets with pepsin buffer several times and after the addition of 1 ml of the SDS-sample buffer (2% SDS, 8 *M* urea, 5% β-mercaptoethanol, 50 m*M* TRIS-HCl pH 7.5), the pellet was sonicated for 5 min and centrifuged. Two hundred microliters of the supernatant was mixed with 200 μl of solution containing 0.38 *M* Na_2CO_3, 5% sodium deoxycholate and 0.01 *M* of NaOH, to which 100 μl of 2 *M* iodoacetate and 2 *M* NaOH was then added. The solution was allowed to stand for 30 min at room temperature and the protein content was determined by the method of Lowry et al. (1951). For total protein, 50 mg of rice powder was suspended in 1 ml of the SDS-sample buffer, sonicated for 5 min on ice and centrifugated. The protein extraction procedure was repeated three times. Two hundred microliters of the supernatant was used for protein determination as described above.

Results and discussion

The three rice mutants, *CM 21, CM 1675* and *CM 1834,* were known to have different glutelin and prolamin contents when compared to wild types, as well as different polypeptide compositions in a molecular size range of 10, 13 and 16 kilodaltons (Kumamaru et al. 1988). To better clarify these differences, we isolated and purified PBs from each mutant by sucrose density gradient centrifuga-

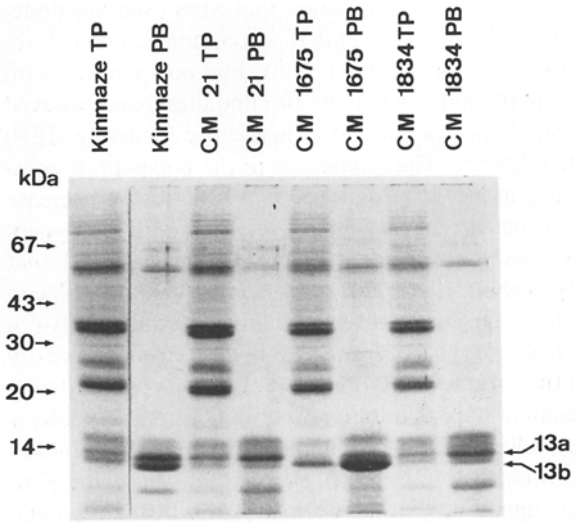


Fig. 1. SDS-PAGE analysis of the proteins contained in protein body-I purified from the starchy endosperm. *TP* – total proteins. *PB* – protein body-I. *13a* – a large component of 13-kilodalton prolamin polypeptide. *13b* – a small component of 13-kilodalton prolamin polypeptide. One hundred micrograms of protein was used for the electrophoresis

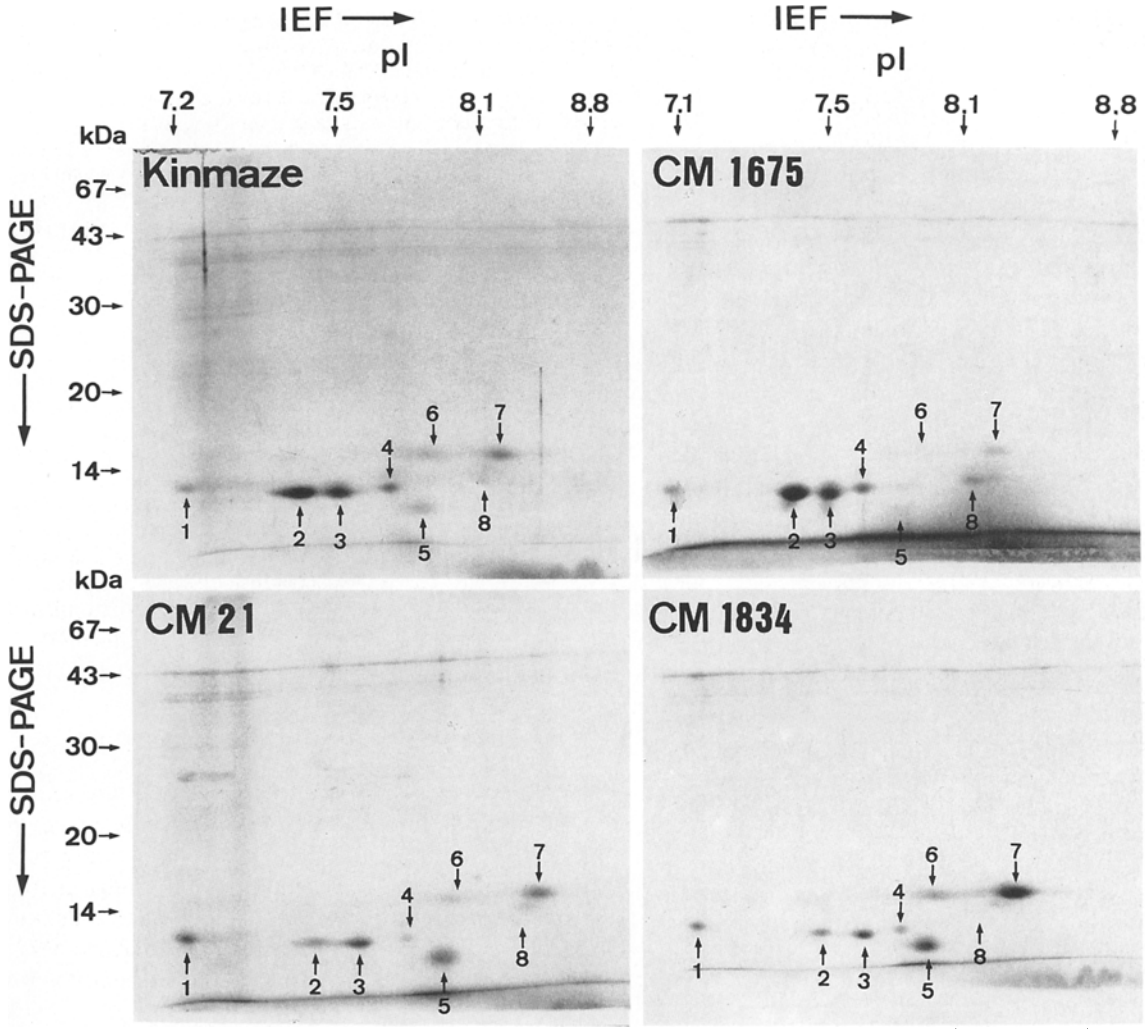
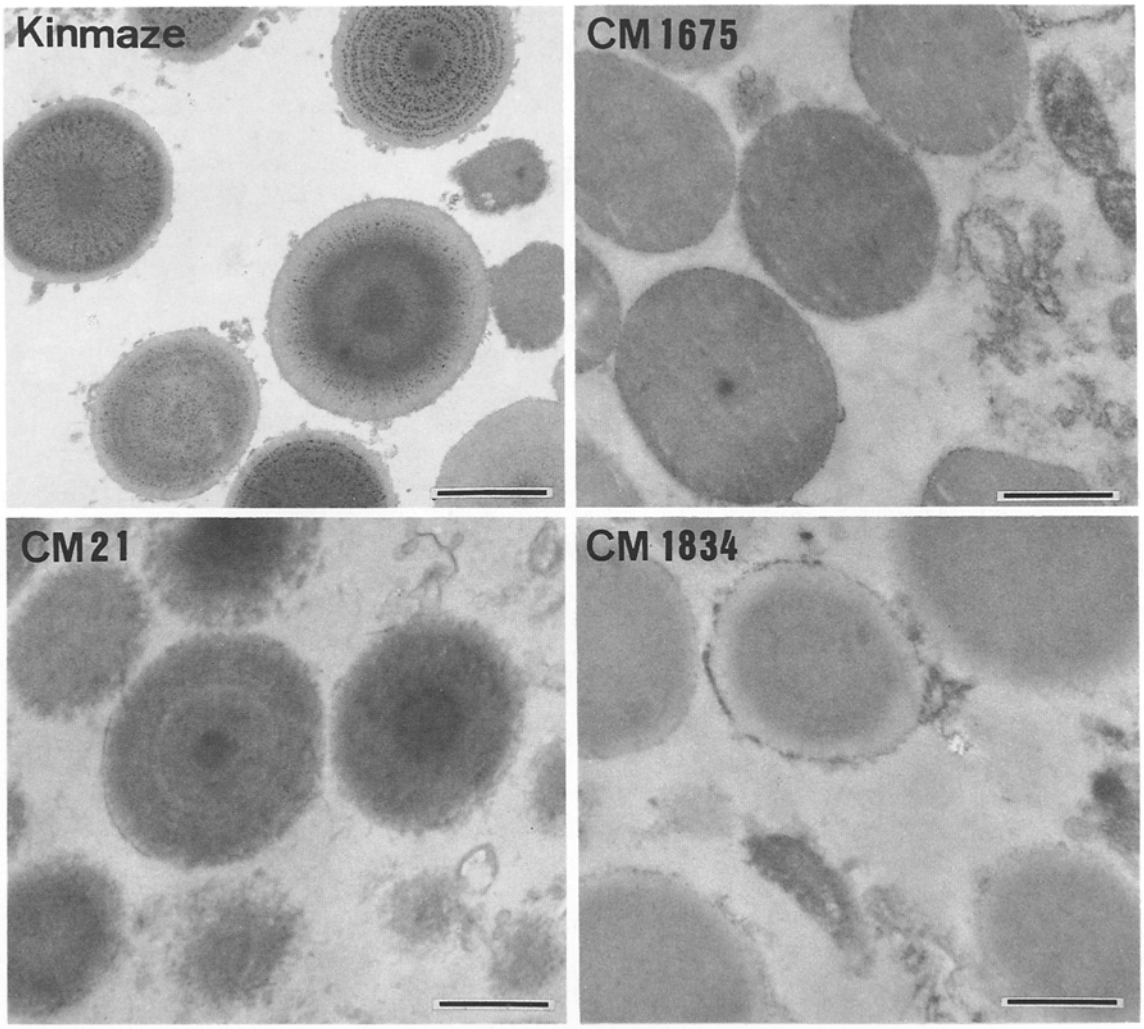


Fig. 2. Two-dimensional gel electrophoretic analysis of the proteins containing in protein body-I purified from the starchy endosperm. Three hundred micrograms of the protein was used for the IEF electrophoresis

tion and pepsin-digestion treatment. Figure 1 shows the profiles of SDS-PAGE analysis polyacrylamide gel electrophoresis of polypeptides in purified pepsin-indigestible PB from Kinmaze and mutants. Polypeptides in PB-I of Kinmaze consist mainly of 13a and 13b polypeptides with lower amount of 10- and 16-kilodalton polypeptides. In the purified PB-I of *CM 21* and *CM 1834*, 13b polypeptide showed a sharp decrease, while in *CM 1675* the 13a and 10-kilodalton polypeptides decreased remarkably. It was, thus, confirmed that the differences between mutants and wild type in polypeptide composition observed by SDS-PAGE of the whole grains (Kumamaru et al. 1988) is still retained when the polypeptide composition present in the PB-I of each mutant is considered.

Figure 2 shows the profiles of two-dimensional gel electrophoretic analysis of proteins from PB-I. Eight clearly defined polypeptides were separated at different pI in Kinmaze. Most of these were located in the pI range from 7.0 to 8.5, indicating that the polypeptides present in PB-I are relatively basic. This analysis confirms that the SDS proteins with MMr of 13 and 16 kilodaltons consist, respectively, of four (nos. 1, 2, 3 and 4) and two polypeptides (nos. 6 and 7), while the SDS 10-kilodalton polypeptide generated in two-dimensional electrophoresis only the polypeptide no. 5. In *CM 21* and *CM 1834*, polypeptides nos. 2, 3, and 4 were weaker than those of Kinmaze. The polypeptides nos. 2, 3 and 4 showed stronger intensities in *CM 1675* than in the reference variety, while polypeptide no. 5 appeared quite faint along with weak intensities for polypeptides nos. 6 and 7. It was also clearly seen that polypeptides nos. 5 and 7 increased in *CM 1834*.

Electron microscopic observations demonstrated that isolated PBs in *CM 21* and *CM 1834* have quite similar lamellar structures to that of Kinmaze (Fig. 3). In con-



trast, PBs isolated from *CM 1675* did not show any typical lamellar structure. The electron densities of the inner structure in *CM 21* and *CM 1834* were relatively higher than in Kinmaze and *CM 1675*. These characteristic structures of purified PBs were also observed in PBs in the mature starchy endosperms of each mutant, confirming that the structural differences found between mutants and Kinmaze did not derive from artifacts arising during purification. The observations of characteristic structures of PB in *CM 21* and *CM 1834* suggest the presence in these mutants of polypeptides which have a high affinity to electron staining reagents in the inner portion of PB leading to the observed morphological changes. Evidence supporting this is as follows.

First of all, the SDS 10-kilodalton, 13a and 16-kilodalton polypeptides present in PB-I are extractable only with 60% n-propanol in the presence of reducing agents (Ogawa et al. 1987). This means that they are probably associated to each other through disulfide bonding. Second, recent studies by Hibino et al. (1989) have shown that the 10- and 16-kilodalton polypeptides have a high methionine content. Further, Masumura et al. (1989), by determining the amino acid sequence of the 10-kilodalton polypeptide deduced from nucleic acid sequences of cDNA clones encoding it, have found that about 30% of the total amino acid content of the 10-kilodalton polypeptide consisted of methionine and cysteine. Therefore, it is likely that the 10- and 16-kilodalton polypeptides exist in the inner portion of PB-I and are associated with other prolamin polypeptides through disulfide bonding. From these results, it is concluded that the differences in protein composition of starchy endosperm, as seen in mutants (Kumamaru et al. 1988), arise from the differences in the composition of polypeptides present in PB-I. It is also concluded that the 10- and 16-kilodalton polypeptides affect the lamellar structure formation of PB-I, and that they play a role in the association of polypeptides present in PB-I.

Table 1. Contents of pepsin-digestible and -indigestible proteins in rice starchy endosperm of three mutants compared to wild type

Plant materials	Protein content (mg/100 mg polished rice)				Ratios[b] of PB-II/PB-I
	Salt-soluble	Pepsin-indigestible	Pepsin-digestible[a]	Total protein	
Kinmaze	1.40	1.57	4.50	7.47	2.9
CM 21	1.44	1.91	7.11	10.46	3.7
CM 1675	1.38	1.50	4.63	7.51	3.1
CM 1834	1.45	1.76	5.87	9.08	3.3

[a] The content was determined by subtracting the sum of pepsin-indigestibles and salt-soluble proteins from total proteins
[b] The value was calculated from the proportion of the content of pepsin-indigestible proteins to that of pepsin-digestible proteins

To evaluate the content of proteins in PB-I and PB-II in mutants, the content of pepsin-indigestible proteins of mature endosperm of Kinmaze and mutants were determined. The content of pepsin-indigestible protein was determined by using pepsin treatment as described in the text, while the content of pepsin-digestible protein was obtained by subtracting the sum of pepsin-indigestible and salt-soluble protein from total protein. The content of pepsin-indigestible and -digestible proteins can be considered, respectively, as the content of proteins in PB-I and PB-II. The mutant contents of salt-soluble proteins were similar to wild type. The pepsin-digestible and -indigestible proteins varied consistently in *CM 21* and *CM 1834* compared to Kinmaze. Pepsin-digestible proteins in *CM 21* and *CM 1834* were, respectively, about 60% and 30% higher than that of Kinmaze, while pepsin-indigestible proteins also increased more – 20% and 10%, respectively – compared to that of Kinmaze. The ratios of PB-II/PB-I proved that the increment of pepsin-digestible proteins in *CM 21* and *CM 1834* were higher than those of pepsin-indigestible proteins. It is concluded that *CM 21* and *CM 1834* are mutants in which the contents of proteins in PB-II and PB-I increased along with the different composition of PB-I polypeptides. In contrast, the contents of pepsin-digestible and -indigestible proteins in *CM 1675* were the same amount as Kinmaze. Therefore, *CM 1675*, in spite of its similar protein content in PB-I and PB-II with Kinmaze, has a simplified polypeptide composition of PB-I.

Kumamaru et al. (1988) determined the content of glutelin and prolamin in mutants by using the solvent extraction method, demonstrating that extracted glutelin and prolamin contents in *CM 21* and *CM 1834* were higher than those of Kinmaze. Higher contents of extracted glutelin and prolamin in *CM 21* and *CM 1834* are consistent with increase in the content of PB-I and PB-II proteins as described in Table 1. However, in *CM 1675* the extracted prolamin content was higher than that of Kinmaze, although the content of proteins in PB-I was similar to that of Kinmaze. Two conjectures present themselves: either the content of propanol-extractable 13-kilodalton prolamin polypeptide in *CM 1675* is higher than that of Kinmaze, or the deficiency of 10- and 16-kilodalton polypeptides in *CM 1675* results in the frail core structure of PB-I and, thus, 13-kilodalton prolamin polypeptides are easily extracted with propanol solution. The first conjecture has been eliminated by the determination of the 13b prolamin polypeptide by enzyme-linked immunosorbent assay using specific antibody raised against purified 13b polypeptide, which demonstrated that the 13b polypeptide content in *CM 1675* is similar to that of Kinmaze (data not shown). Therefore, we propose that the 10- and 16-kilodalton polypeptides are closely associated with the PB-I structure. Although the relationship between the formation of PB-I structure and the presence of 10- and 16-kilodalton polypeptides is unknown, in view of the protein composition and the inner structure of PB-I in *CM 1675*, it may be assumed that the 10- and 16-kilodalton polypeptides play an important role in the formation of the rigid structure of PB-I.

It is interesting to note that none of the individual polypeptides seen in PB-I of Kinmaze was totally absent in any mutant. Instead, in these mutants, the contents of individual polypeptides are relatively high and low, that is, these polypeptides in PB-I differ only in quantity. This suggests that the mutations are related not to the structural genes but to the controlling genes of protein synthesis in PB-I, hence they can be used as the potential genetic resources for breeding high-glutelin rice with the improved digestibility of PB-I.

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