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Inheritance of alleles for glutelin α-2 subunit genes in rice and identification of their corresponding cDNA clone

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Abstract Rice glutelins consist of acidic (α) and basic (β) subunits which are further separated into three polypeptide components assigned as α-1, α-2, and α-3 subunit components and β-1, β-2 and β-3 subunit components. Nine rice mutant lines with a decreased amount of the glutelin α-2 subunit component (α-2L) were obtained by screening about 6,800 potential mutant lines derived from the fertilized egg treatment with N-methyl-N-nitrosourea (MNU) using the SDS-PAGE method. The mutants were classified into three types of the increased α-1 subunit (α-1H/α-2L), the decreased β-2 subunit (β-2L/α-2L) and the increased α-3 subunit (α-3H/α-2L) represented by EM278, CM1707 and EM659, respectively. Iso-electric focus (IEF) analysis revealed that all of the mutants had an extremely low amount of a polypeptide with a 6.71 pI value, whereas a polypeptide with either a 6.50 pI value or with a 6.90 pI value increased significantly in α-1H/α-2L mutants or in α-3H/α-2L mutants, respectively. The β-2L/α-2L mutants had a decreased amount of a basic polypeptide with a 8.74 pI value. Genetic analysis revealed that the three types of mutants were controlled by a single incomplete dominant gene respectively, and the three are alleles. The gene was temporarily named *glu4*, which was found to be located on chromosome 1 linked with the *eg* and *spl6* genes. Two-dimensional electrophoresis analysis revealed that the *glu4* encoded polypeptides of pI 6.71/α-2 and pI 8.74/β-2. Amino acid sequence analysis suggested that the mutated acidic polypeptide was the product of a GluA subfamily gene. Northern and RT-PCR analyses revealed that *glu4* corresponded to the GluA-1 gene.

Keywords Rice glutelin · Mutant · Alleles · Gene function

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Introduction

Rice is not only one of the most important food resources but also one of the most important protein sources for humans worldwide, especially for those in developing countries where animal proteins are not easily available. Therefore, it is important to improve the quality and nutritional value of the protein in rice. Unlike other cereal crops those deposit prolamine as their main storage protein, rice stores a high content of glutelin accounting for more than 70% of total proteins on a weight basis (Ogawa et al. 1987; Li and Okita 1993). Compared to prolamine, glutelin is more easily digestible and contains a higher amount of the essential amino-acid lysine (Tanaka et al. 1975; Ogawa et al. 1987; Resurreccion et al. 1993). Since glutelin is the pre-dominate storage protein in rice seed, any change of this protein will certainly alter the seed quality.

It was confirmed that rice glutelin is synthesized as proglutelin with *Mr* 57,000 (57 kDa) on the membrane-bound polysomes, and that the proglutelin is processed proteolytically into acidic (α) and basic (β) subunits with *Mr* 40,000 (40 kDa) and *Mr* 20,000 (20 kDa), respectively, within the protein storage vacuole called the protein body II (PB II) (Yamagata et al. 1982; Zhao et al. 1983; Furuta et al. 1986; Krishnan and Okita 1986; Yamagata and Tanaka 1986). Kagawa et al. (1988) fractionated the acidic and basic subunits into three bands each, namely α-1, α-2 and α-3, and β-1, β-2 and β-3, respectively.

Wen and Luthe (1985) reported that rice glutelin polypeptides were separated into at least sixteen acidic subunits and nine basic subunits, respectively, by using isoelectric focusing (IEF), suggesting that the IEF glutelin bands correspond to the products of a system of structural genes. The N-terminal and internal amino-acid sequences of the acidic polypeptides have been determined by Hirano et al. (1991) and Komatsu et al. (1993).

Rice seed-storage glutelin is of high homology with the 11S legumin. The inheritance of soybean storage proteins has been intensively studied and many alleles have been reported (Kitamura et al. 1984; Davies et al. 1985; Scallon et al. 1987; Choi et al. 1989a, b; Diers et al. 1994). In rice, at least five glutelin genes that concern the genetic regulatory mechanism on glutelin post-translation processing, transportation to the protein body and deposition, have been identified (Kumamaru et al. 1987; Satoh et al. 1994, 1995, 2000). However, there are few formal reports on the inheritance of glutelin structure gene (Iida et al. 1997).

Rice glutelin is estimated to be encoded by about ten copies of genes per haploid genome (Okita et al. 1989; Takaiwa and Oono 1991). The genes are co-expressed in rice endosperm; however, their contributions to the glutelin content are different since the amounts of individual polypeptide observed by gel electrophoresis are not even. Thus far, at least eight glutelin cDNA or genomic DNA clones have been isolated and sequenced (Takaiwa et al. 1987, 1991; Masumura et al. 1989; Okita et al. 1989). These clones, with two pseudo-genes among them, were classified into two subfamilies of GluA and GluB (Takaiwa et al. 1987, 1991). The GluA subfamily is composed of at least four members (A1, A2, A3 and A4), which show 73–96% sequence identity to each other (Takaiwa et al. 1987, 1991; Okita et al. 1989). The GluB subfamily also consists of four members (B1, B2, B3 and B4) whose coding regions show 80–88% sequence identity to each other (Masumura et al. 1989; Takaiwa et al. 1991); whereas the coding sequences between the subfamilies share 60–65% identity at the amino-acid level. However, the functions of these cloned genes have not been clearly identified though it was demonstrated that *glu1* was a member of the GluB subfamily, and *glu2* and *glu3* belong to the GluA subfamily (Iida et al. 1997). The further effective use of these cloned genes necessitates the clarification of their functions.

Mutations that affect seed storage proteins have played important roles in illustrating important features associated with the study of seed proteins and their inheritance. Properties of plant genomes that might otherwise be overlooked are often revealed by mutations. Recently, we found various kinds of rice mutants lacking the glutelin α-2 subunit by screening the progenies from mutation treatment with methyl-nitrosourea (MNU). In this paper, we report the glutelin characterization, the results of genetic analysis of the mutants, the relationships among the mutated genes and the loci of the genes to illustrate the variability within the locus. We also identify the genes, their products of the glutelin subunits and their correspondent cDNA clone in order to reveal the glutelin gene functions.

Materials and methods

Plants

About 6,800 potential mutant lines generated from the progenies of fertilized egg cells of rice (*Oryza sativa* L. cv Kinmaze) treated with MNU were used in this study.

SDS-PAGE

Electrophoresis was carried out by using the discontinuous buffer system of Laemmli (1970) on a slab gel containing a linear gradient of 15–25% acrylamide and 0.05–0.67% bisacrylamide concentrations. The gel was stained by 0.15% coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid after electrophoresis, and de-stained by 10% acetic acid in 25% methanol.

Screening of mutants

Whole proteins were extracted from one grain of each mutant line and the original cultivar. The grain was crushed by pliers, placed in a micro test tube, added with 0.7 ml of sample buffer containing 125 mM Tris-HCl, pH 6.8, 4 M urea, 4% SDS and 5% 2-mercaptoethanol, and shaken over night after vortexing. After centrifugation (5,000 rpm, 15 min) 4.5 μl of the supernatant was applied to SDS-PAGE. Mutants for glutelin were determined by comparing the SDS-PAGE profiles with that of Kinmaze.

Crosses

The mutant lines were crossed with Kinmaze to investigate the inheritance of the mutated characters. Mutant lines were crossed reciprocally to test the allelism of the mutated genes. A series of trisomic plants concerned with chromosomes No. 4 to No. 12, derived from a desynaptic mutant induced from Kinmaze, and marker gene lines were crossed with mutant lines to determine the chromosome on which the mutated genes were located and where the gene loci were. The proteins of F_1, F_2 and F_3 seed of all crosses were analysed by SDS-PAGE.

Iso-electric focusing (IEF)

Horizontal slab IEF gels were prepared and focused according to Brinegar and Peterson (1982). The gel contained 6 M urea, 4% acrylamide, 2% NP-40, 2% Ampholine with the rate of pH 3.5–10.0:pH 6.0–8.0:pH 8.0–10.5 = 1:1:1. The glutelin extracted from rice endosperm according to Kumamaru et al. (1987) was dissolved in O'Farrell solution containing 8.5 M urea, 2% Nonidet P-40 and 5% 2-mercaptoethanol (O'Farrell 1975) and subjected to IEF electrophoresis according to the procedure of Wall et al. (1984). After IEF electrophoresis the gel was incubated in 15% trichloroacetic acid (TCA) for 20 min, stained with 0.15% commassie brilliant blue R-250 in 50% ethanol, 10% acetic acid, and de-stained with 25% ethanol and 10% acetic acid.

Two-dimensional electrophoresis

The bands of IEF, the first-dimensional electrophoresis, were cut from the gel and incubated in 125 mM Tris-HCl, pH 6.8, containing 4% SDS for 2 h and loaded onto SDS-PAGE, the second-dimensional electrophoresis.

N-terminal amino-acid sequence

After separation by two-dimensional electrophoresis (2-DE), proteins were electro-blotted onto a PVDF membrane and detected by CBB staining. The N-terminal pyroglutamic acid was removed by pyroglutamyl peptidase (Hirano et al. 1991). The membrane was then washed with de-ionized H_2O, dried and subjected to the gas-phase protein sequencer (476A, Applied Biosystems). Edman degradation was performed according to the standard program supplied by the manufacturer.

Internal amino-acid sequence analysis

The appropriate band of 2-DE was cut out with a razor blade, soaked with 125 mM of Tris-HCl, pH 6.8, containing 0.1% SDS and 1 mM EDTA for 30 min. The gel slice was placed in the sample-well of the tricine-SDS-polyacrylamide gel (Schagger and Jagow 1987) and overlaid with *Staphylococcus aureus* V8 protease. The polypeptide was digested in the stacking gel during electrophoresis as described by Cleveland et al. (1977). After electrophoresis, the glutelin digests were electro-blotted onto the PVDF membrane, dried and subjected to the gas-phase sequencer.

RNA preparation and Northern analysis

Rice developing seeds were harvested 10–14 days after flowering and frozen in liquid nitrogen. Total RNA was extracted by using Isogen (Nippongene Ltd.) according to the manufacturer's instructions. Total RNA (10 μg) was resolved on a 0.6% agarose-formaldehyde gel and transferred onto a nylon membrane (Hybond N+). Rice glutelin cDNAs which belonged to GluA and GluB subfamilies were labeled and used for hybridization as probes. cDNA labeling, hybridization, washing and signal detection were performed by using the ECL system (Amersham) according to the manufacturer's instructions.

RT-PCR

First-strand cDNA was generated by using the T-primed first-strand system (Pharmacia) according to the manufacturer's instructions. The generated first-strand cDNA was used as a template for PCR. The GluA-1 specific pairs of fragments were used as forward (5′-TCCAAGAGAATGCAGGTTCG-3′) and reverse (5′-AGGTCAGAAAGCTGATTACTA-3′) primers. Thermal cycling using *Taq* polymerase was as follow: denaturing at 94 °C for 4 min, followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 2 min.

Results

Identification of rice glutelin mutants

Total seed proteins from about 6,800 potential mutant lines derived from the fertilized egg-cell treatment with MNU (Satoh and Omura 1979) of the rice cultivar of Kinmaze were analyzed by SDS-PAGE. Using the 15–25% linear gradient acrylamide gel, rice glutelin acidic subunits were clearly separated into three bands that were named α-1, α-2 and α-3 with an *Mr* of 39–37 kDa (Fig. 1). The glutelin basic subunits were also separated into three bands of β-1, β-2 and β-3 with an *Mr* of 23–22 kDa, but the resolution of the basic subunit was not as clear as that of the acidic subunit. By comparing with the SDS-PAGE profile of Kinmaze, nine

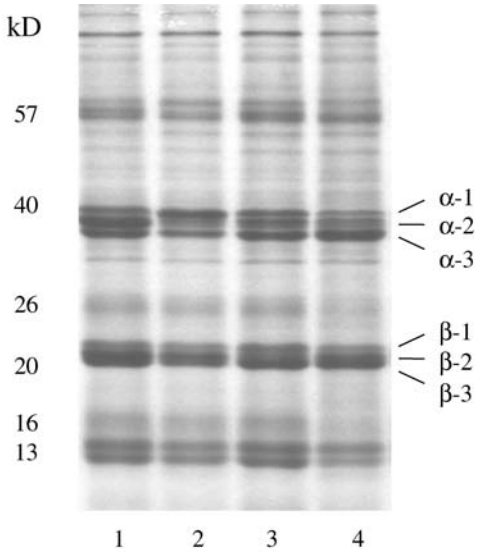

Fig. 1 SDS-PAGE analysis of the seed proteins in mutant lines with a decreased glutelin α-2 subunit. All of the mutants showed a decreased amount of the α-2 subunit (α-2). Meanwhile EM278 showed an increased amount of the α-1 subunit (α-1H/α-2L type, *lane 2*), CM1707 showed a decreased amount of the β-2 subunit (β-2L/α-2L type, *lane 3*), and EM659 showed an increased amount of the α-3 subunit (α-3H/α-2L type, *lane 4*). *Lane 1*: Kinmaze

mutant lines with a decreased amount of the glutelin α-2 subunit (α-2L) were obtained. According to their SDS-PAGE profiles, the nine mutants were classified into three types: two lines (EM278 and TCM2622) with a high amount of α-1 subunit (α-1H/α-2L type, Fig. 1, lane 2), three lines (CM1707, TCM1170 and TCM1224) with a decreased β-2 subunit (β-2 L/α-2L type, Fig. 1, lane 3) and four lines (EM659, CM439, TCM221 and TCM222) with a high amount of the α-3 subunit (α-3H/α-2L type, Fig. 1, lane 4).

Genetic analysis of mutants

Different mutant lines within the same type were crossed with each other, and the phenotypes of the F_1 and F_2 seeds were investigated. All F_2 seeds of the crosses within each type did not show any segregation. Hence, each type of the mutants was considered to be controlled by the same gene. One line from each type was crossed with Kinmaze reciprocally, and the F_1 and F_2 seeds were investigated on the α-2 subunit by SDS-PAGE. In the crosses between Kinmaze and EM278 (α-1H/α-2L), the stain densities of the α-2 band were in the order of Kinmaze, F_1 seed of the cross between Kinmaze (♀) and EM278 (♂), F_1 seed of the cross between EM278 (♀) and Kinmaze (♂) and EM278 (Fig. 2a). The phenotypes of F_2 seeds were classified into four types, two parent types and two reciprocal F_1 types mentioned above (Fig. 2) named Kinmaze type, α-2L type, near the Kinmaze type and near the α-2L type. In the crosses be-

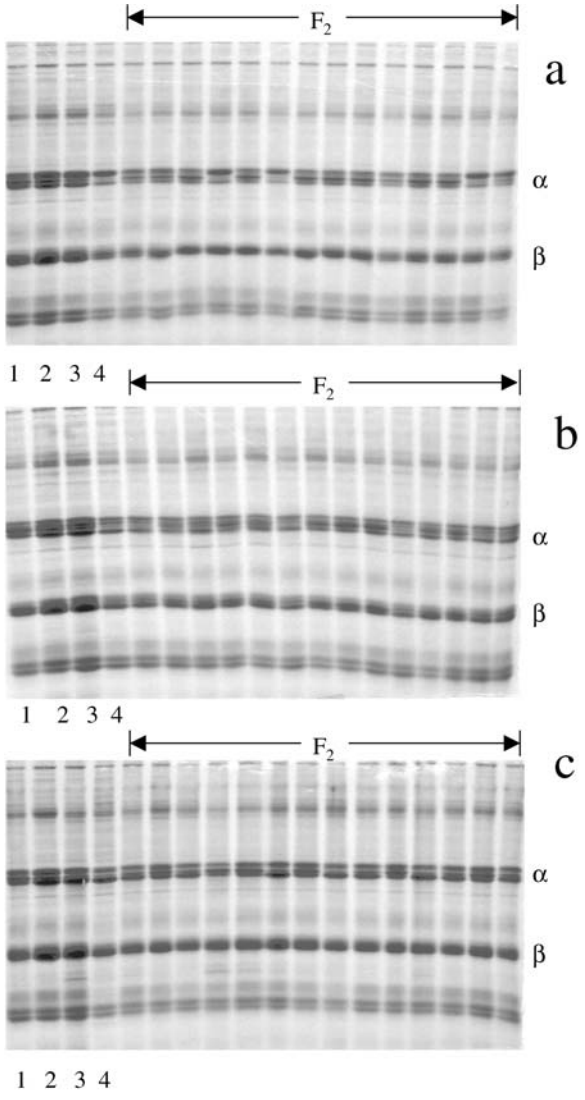

Fig. 2a–c SDS-PAGE phenotypes of F_1 and F_2 seeds from the crosses between Kinmaze and the mutants. *Lane 1* Kinmaze; *lanes 2 and 3* reciprocal F_1 seeds of the cross between Kinmaze and the mutants; *lane 4* the mutant. F_2: the F_2 generation. **a** Cross between Kinmaze and EM278; **b** Cross between Kinmaze and CM1707; **c** Cross between Kinmaze and EM659

tween Kinmaze and CM1707 (β-2L/α-2L), and Kinmaze and EM659 (α-3H/α-2L), the phenotypes of α-2 subunits in F_1 and F_2 seeds were the same as those in the cross between Kinmaze and EM278 (Fig. 2b and c). The segregation mode in the F_2 seeds of the crosses between Kinmaze and the mutants is shown in Table 1. The result that the segregation ratios fitted the expected ratio of 1:1:1:1 showed that the three mutated characters were controlled by a single incomplete dominant gene, respectively.

Rice endosperm cells are triploid since they contain two copies of the maternal and one copy of the paternal genes. In the case of the mutant for the structural gene coding the polypeptide, the gene dosage effect should appear in the polypeptides of the F_1 and F_2 seeds. The gene dosage effect has been reported to occur in the F_1 and F_2 seed storage protein in barley (Doll 1980), wheat (Dhaliwal 1977; Burnouf et al. 1983), maize (Valentini et al. 1979; Soave et al. 1981), rice glutelin (Iida et al. 1997) and rice globulin (Iida et al. 1998). In this study, the gene dosage effect was observed clearly on the α-2 subunit in both F_1 and F_2 seeds. These facts indicated that α-2L was encoded by a structural gene.

It must be noted that the character of α-1H did not separated with α-2L in the F_2 generation of the cross between EM278 and Kinmaze, i.e. the α-1H was always accompanied by α-2L. So did the β-2L between CM1707 and Kinmaze, and the α-3H between EM659 and Kinmaze, respectively. These results indicated that the character pair in each type of mutant was encoded by the same gene, respectively.

Allelic relationship among the genes encoding α-2L

The mutant lines were crossed with each other reciprocally and the expression of the α-2 subunit in the F_1 and F_2 seeds was investigated. Figure 3a shows the SDS-PAGE band patterns of the F_1 and F_2 seed protein from the cross between EM278 and EM659. All the reciprocal F_1 and F_2 seeds showed the α-2L character. The wild-type of the α-2 subunit did not appear at all. The same results were obtained from the crosses between EM278 and CM1707, and between CM1707 and EM659 (Fig. 3b and c). These results indicated that the α-2L in the three mutants was the result of mutation at the same locus.

Though the segregation of α-2L was not found in the progenies of the crosses among the mutants, the segregation on other subunit was observed. In the cross between EM278 and EM659, the phenotypes of the reciprocal F_1 seeds showed intermediate types but were near their female parents on the α-1 and α-3 subunits, i.e. the staining intensities of the α-1 band were in the order of

Table 1 Segregation of glutelin types in F_2 from the crosses between mutants and Kinmaze

Crosses ($P_1 \times P_2$)	Segregation in F_2				$\chi^2(1:1:1:1)$
	$P_1P_1P_1$	$P_1P_1P_2$	$P_1P_2P_2$	$P_2P_2P_2$	
Kinmaze × EM278	24	23	28	25	0.56
Kinmaze × CM1707	21	26	26	26	0.92
Kinmaze × EM659	24	23	26	27	0.40

Table 2 Segregation of glutelin types in F_2 from the crosses between mutants and the original cultivar

Crosses ($P_1 \times P_2$)	Segregation in F_2				$\chi^2(1:1:1:1)$
	$P_1P_1P_1$	$P_1P_1P_2$	$P_1P_2P_2$	$P_2P_2P_2$	
EM278 × CM1707	26	23	26	25	0.24
CM1707 × EM659	24	25	22	23	0.22
EM278 × EM659	26	23	25	26	0.16

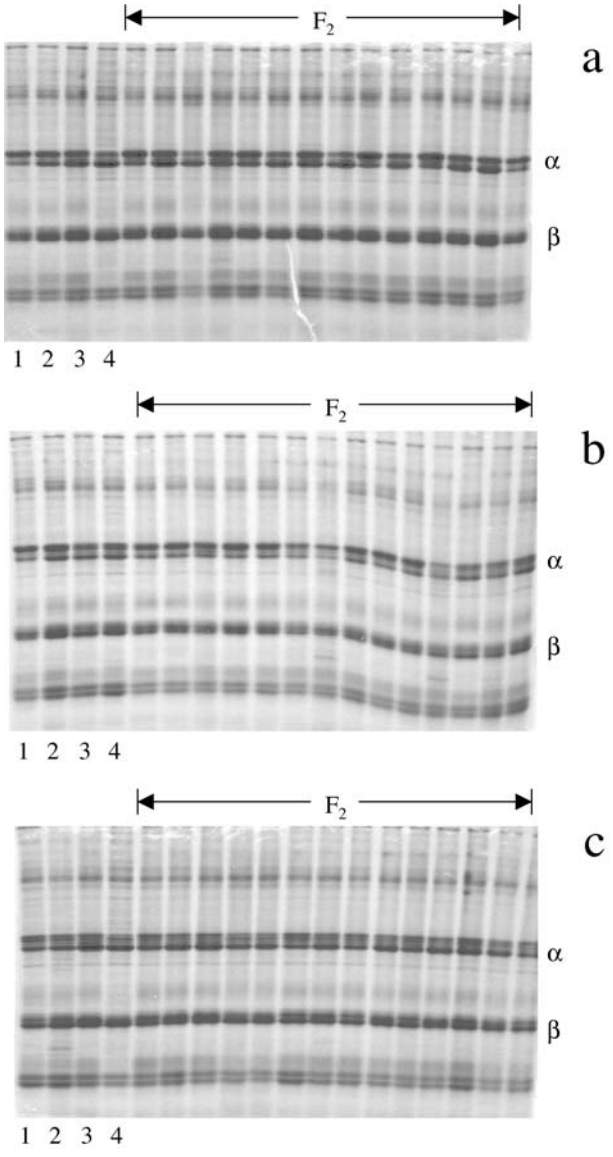

Fig. 3a–c SDS-PAGE phenotypes of F_1 and F_2 seeds from the cross among mutants. *Lanes 1 and 4* mutants; *Lanes 2 and 3* reciprocal F_1 seeds; F_2:F_2 seeds. **a** Cross between EM278 and EM659. **b** Cross between EM278 and CM1707. **c** Cross between CM1707 and EM659

EM278, the F_1 seed of EM278 as female parent, the F_1 seed of EM659 as female parent and EM659; while that of the α-3 bands were opposite (Fig. 3a). The phenotypes of the F_2 seeds could also be classified into four types, two parent types and two F_1 types. In the cross combinations between EM278 and CM1707, and CM1707 and EM659, similar results were also obtained (Fig. 3b and c). The segregation modes in the F_2 seeds of the reciprocal crosses between mutants are shown in Table 2. The segregations fitted the expected ratio of 1:1:1:1 in all crosses. The genes in EM278, CM1707 and EM659 were designated tentatively as *glu4a*, *glu4b* and *glu4c*, respectively, in comparison with the *glu1*, *glu2* and *glu3* genes reported by Iida et al. (1997).

It is worth noting that the characteristics of α-1H in the *glu4a* mutant of EM278, and α-3H in the *glu4c* mutant of EM659, were expressed simultaneously in the F_1 generation of the crosses between the two mutants (Fig. 3a). Similar phenomena were also observed in the other crosses between EM278 and CM1707, and CM1707 and EM659 (Fig. 3b and c). These results indicated that the alleles of *glu4* were co-dominant.

Mapping of the mutated gene

A series of trisomics with an extra chromosome from No. 4 to No. 12 were crossed with EM659 carrying *glu4c*. The segregations of α-2 subunits in F_2 seeds were investigated. Trisomic segregation was not found in all crosses, meaning that the gene was located on neither of these chromosomes. Furthermore the mutant, EM659, was crossed with a series of marker lines concerning chromosomes 1, 2 and 3. The *glu4c* gene was found to be linked with the *spl6* and *eg* genes which were located on chromosome 1, with recombination values of 17.2% and 24.2%, respectively (Table 3). Based on that the recombination values of 7% between the *spl6* and *eg* genes (Kinoshita 1995), the order of the genes on chromosome 1 was estimated to be *eg–spl6–glu4c* as shown in Fig. 4.

IEF analysis of glutelin α-2L mutants

Glutelin fractions extracted by 1.0% lactic acid were analyzed by the horizontal slab IEF system. In this system, glutelin acidic proteins of Kinmaze were separated into thirteen polypeptide bands with the iso-electric point (pI) ranging from pI 6.30 to pI 7.52, and the basic subunit proteins were separated into eleven polypeptide-bands from pI 8.13 to pI 9.14 (Fig. 5), respectively. The staining intensities of these bands were not equal, reflecting the differences of expression on individual glutelin polypeptides. On the acidic side, six acidic subunit bands of pI 6.59, pI 6.71, pI 6.82, pI 6.90, pI 7.19 and pI 7.38 were stained much more intensively than the others. On the basic side, six basic subunit bands of pI 8.24, pI 8.50, pI 8.58, pI 8.74, pI 8.78 and pI 8.86 were also stained

Table 3 Linkage analysis among *glu4c*, *spl6* and *eg* genes

Crosses	Segregation in F_2						Cv (%)
	+ ++	+ +*glu4c*	+ *glu4cglu4c*	m[a] ++	m +*glu4c*	m *glu4cglu4c*	
spl6 × *glu4c*	16	56	37	18	8	1	17.2
eg × *glu4c*	18	55	36	16	9	2	24.2

[a] m: recessive marker genes

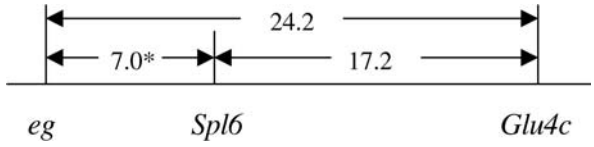

Fig. 4 Linkage map among the *glu4c, spl6* and *eg* genes on chromosome 1. *: Kinoshita (1995)

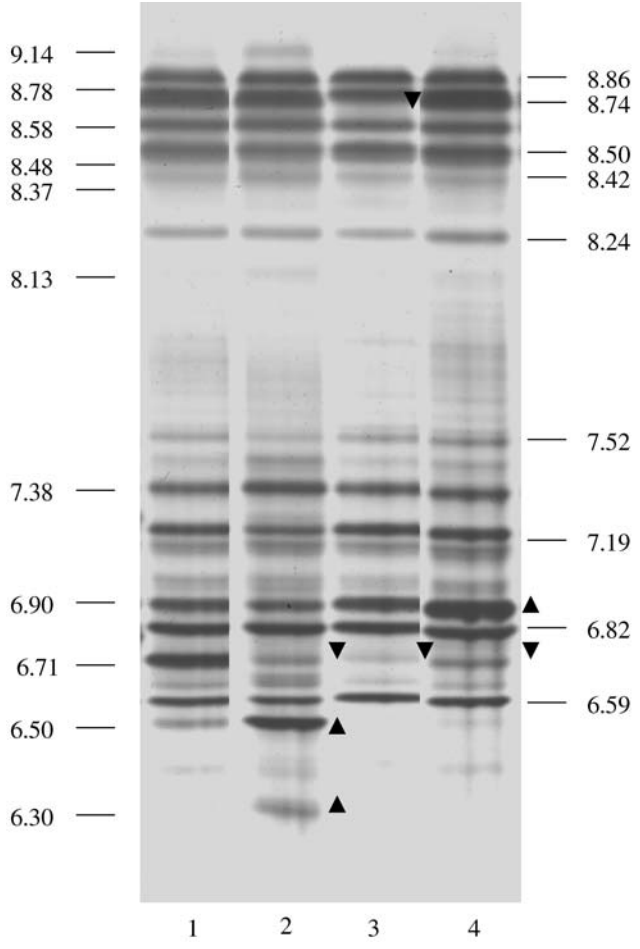

Fig. 5 IEF analysis of rice glutelin α-2L mutants. *Lane 1* Kinmaze; *lane 2* EM278; *lane 3* CM1707; *lane 4* EM659. ▼: decreased, ▲: increased

much more intensively than the others. The pI 6.71 band greatly decreased in common in all of the α-2L mutants, while the staining profile of the other bands varied extremely among the mutant types (Fig. 5). In the α-1H/α-2L mutant of EM278, the pI 6.30 and pI 6.50 bands obviously increased. In the α-3H/α-2L mutant of EM659, the pI 6.90 band increased significantly. No distinct changes were found on the basic side in these two types of mutants. However, in the β-2L/α-2L mutant of CM1707 the pI 8.74 band, on the basic side, obviously decreased. In addition, the pI 6.30 and pI 6.50 bands disappeared from the β-2L/α-2L and α-3H/α-2L mutants.

Two-dimensional electrophoresis analysis of the *glu4b* mutant

Extracted glutelins of Kinmaze and CM1707 were analysed by two-dimensional electrophoresis analysis. The results revealed that in Kinmaze the glutelin acidic bands of pI 6.59 and pI 6.82 fractioned by IEF are components of the α-1 subunit, and those of bands pI 6.71 and pI 6.90, and pI 7.19 and pI 7.38 are components of the α-2 and α-3 subunits, respectively (Fig. 6). Compared to Kinmaze, one glutelin acidic polypeptide of pI 6.71/α-2 and one basic polypeptide of pI 8.74/β-2 decreased greatly in CM1707 (Fig. 6). Rice glutelin is synthesized as a 57 kDa precursor encoded by a structural gene, and then the precursor is processed proteolytically into one acidic and one basic polypeptide (Yamagata et al. 1982). Therefore one glutelin gene must correspond to one acidic and one basic polypeptide. The fact that one acidic and one basic polypeptide decreased simultaneously in the mutant suggested that the two polypeptides were products of the *glu4* gene.

N-terminal and internal amino-acid sequences of the pI 6.71/α-2 polypeptide

To obtain more information about the mutated polypeptides, the polypeptide of pI 6.71/α-2 in Kinmaze was purified and trans-blotted onto the PVDF membrane. After de-blocking with pyroglutamate aminopeptidase the glu-

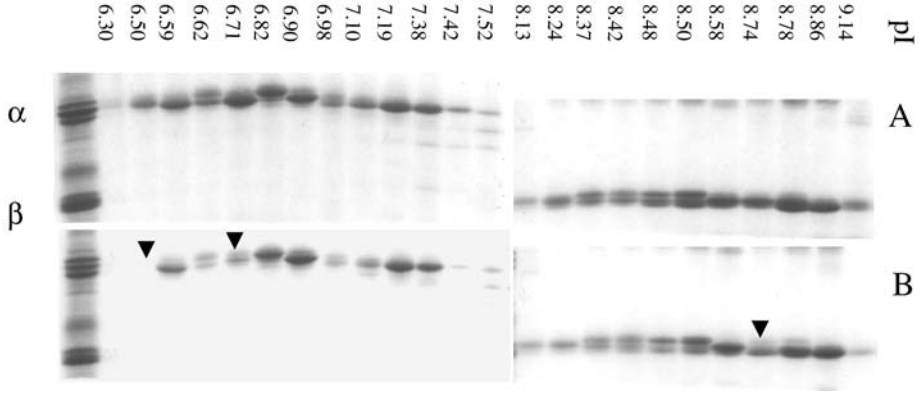

Fig. 6 Two-dimensional electrophoresis analysis of glutelin fractions from Kinmaze and CM1707. **A** Kinmaze; **B** CM1707. ▼: deletion/decreased amount

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                    10          120
Glutelin A-1    <QQLLGQSTSQ------SQSQSQKFKD---
pI6.71/α-2      <QQLLGQSTSQ------SQSQSQKFKD---
Glutelin A-2    <QQLLGQSTSQ------SQSQSHKFKD---
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Fig. 7 Amino-acid sequences of the mutated glutelin polypeptide of pI 6.71/α-2. Glutelin A-1 and Glutelin A-2: the sequences deduced from the nucleotide sequence of the GluA-1 and GluA-2 cDNA clone, respectively. <Q represents pyroglutamic acid

telin acid polypeptides were subjected to the gas-phase protein sequencer to determine the N-terminal amino-acid sequence of the polypeptide. The amino-acid sequences of pI 6.71/α-2 of Kinmaze from the second residue were also determined (Fig. 7). The N-terminal amino-acid residue of these polypeptides was considered to be pyroglutamic acid formed by the cyclization of glutamine or glutamic acid (Komatsu et al. 1993). The N-terminal amino-acid sequence of ten residues showed identity to those putative N-terminal amino-acid sequences deduced from GluA-1 (Gt2) and GluA-2 (Gt1) (Takaiwa et al. 1987; Okita et al. 1989) and the internal amino-acid sequences were 100% homolous with that deduced from GluA-1 cDNA, while only one amino-acid residue was different from that deduced from GluA-2 cDNA, i.e. the 122nd residue of glutamine in GluA-1 with that of histidine in GluA-2 (Takaiwa et al. 1987). This result suggested that the *glu4* gene encoding the pI 6.71/α-2 polypeptide belonged to GluA-1.

Identification of the *glu4* gene and its corresponding cDNA clone

The fact that the acidic polypeptide of pI 6.71/α-2 and the basic polypeptide of pI 8.74/β-2 decreased simultaneously in CM1707 suggested that the expression of the gene (*glu4*) is either suppressed or that the products of the gene are processed after translation due to the abnormal structure caused by mutation. Expression of the mutated gene was examined by Northern-blot hybridization. Only one spot of a possible transcript was detected and it corresponded to an mRNA of about 1.8 kb. When probed with GluA-1 cDNA, the spot of CM1707 (*glu4b*) was tremendously weaker than that of Kinmaze (*glu4*) (Fig. 8A). However when the same membrane was reprobed with GluA-2 cDNA, no difference was found between CM1707 (*glu4b*) and Kinmaze (*glu4*) (Fig. 8B) though the nucleotide sequence of GluA-2 cDNA shared 96% homology with that of GluA-1. Furthermore, no differences were found between CM1707 (*glu4b*) and Kinmaze (*glu4*) when probed with GluA-3, GluB-1 and GluB-2 cDNAs either (data not shown). These results indicated that CM1707 was a GluA-1 gene mutation reflecting that the decrease of pI 6.71/α-2 and pI 8.74/β-2 subunits in CM1707 was caused by abnormal transcription of the GluA-1 gene that prevented it from expressing.

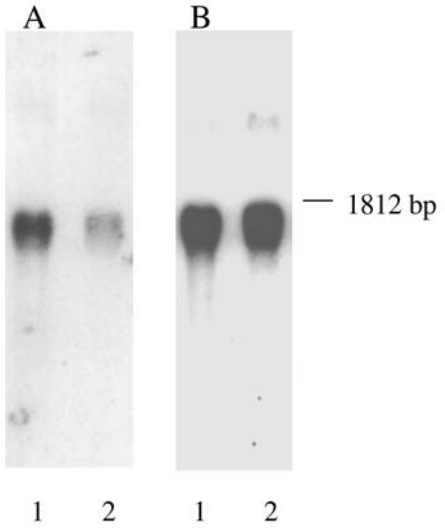

Fig. 8A, B Northern hybridization of *glu4b* mutant. **A** GluA-1 cDNA as a probe; **B** GluA-2 cDNA as a probe. *Lane 1* Kinmaze (*glu4*); *lane 2* CM1707 (*glu4b*). Rice 17S rRNAs (1,812 base pair) were used as a size marker

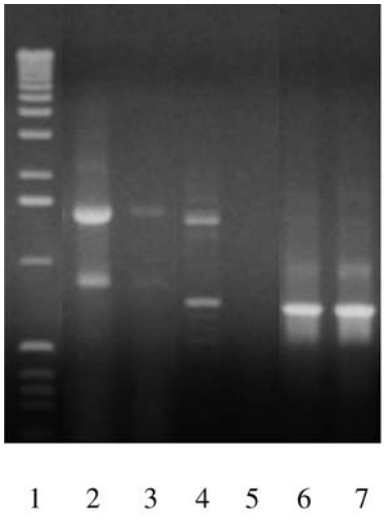

Fig. 9 RT-PCR analysis of *glu4* genes. GluA-1 specific nucleotides were used as primers (*lanes 2 to 5*). PDI specific sequences as primers were used as negative control (*lanes 6 and 7*). *Lanes 2 and 6* first-strand cDNA from Kinmaze was used as template; *lanes 3 and 7* first-strand cDNA from CM1707 was used as template; *lanes 4 and 5* GluA-1 cDNA and GluA-2 cDNA were used as templates, respectively. *Lane 1* was a molecular marker

The result that CM1707 was a GluA-1 mutation was also verified by PT-PCR analysis. Total RNA used for Northern blotting was used as a template to synthesize the first-strand DNA by reverse transcriptase using the T-primed first-strand system (Pharmacia). The resulting first-strand DNA was used as a template to amplify the mutated gene by PCR with GluA-1 specific nucleotide sequences as primers. The PCR product of the expected size was checked by comparing it to that generated with GluA-1 cDNA as a template.

By using the unique nucleotide sequences of GluA-1 as primers, the GluA-1 cDNA amplified successfully, while the amplification of GluA-2 cDNA was not observed (Fig. 9) though the two cDNAs shared 96% homology (Takaiwa et al. 1987; Okita et al. 1989). These results confirmed the specificity of the primers. When the first-strand cDNA of CM1707 and Kinmaze were used as templates, the PCR products of CM1707 decreased tremendously compared to that of Kinmaze (Fig. 9). When the same synthesized first-strand cDNAs were used as a template to amplify the PDI (protein disulfide isomerase) gene, no difference was found between CM1707 and Kinmaze (Fig. 9). These results confirmed that the signal difference between CM1707 and Kinmaze was not caused by cross hybridization, reflecting the fact that the transcription of the mutated gene mRNA was obstructed, which prevented the gene from expression. The results of RT-PCR also indicated that CM1707 was a pre-transcriptional mutation but not a post-transcriptional mutation.

Discussion

Rice glutelin is synthesized as a 57 kDa precursor on membrane-bound polysomes and the precursor is transported to PB II through the Golgi complex. Finally the precursor is processed proteolytically within the PB II, yielding mature glutelin acidic and basic subunits. The genetic regulatory mechanisms on glutelin transportation, deposition and processing have been intensively studied. At least five regulatory genes have been reported and the chromosomal locations of the genes have been identified (Kumamaru et al. 1987; Satoh et al. 1994, 1995, 2000). Iida et al. reported three glutelin genes and mapped them onto chromosome 1, 2 and 10.

In this study, nine allelic mutant lines with a decreased amount of the glutelin α-2 subunit were obtained by screening about 6,800 potential mutant lines derived from the fertilized eggs of Kinmaze treated with MNU. Different from those reported by Iida et al. (1997), the α-2L mutants reported here lack/decrease the same polypeptide of pI 6.71. Simultaneously, the polypeptides of pI 6.50 and pI 6.90 increased significantly in α-1H/α-2L and α-3H/α-2L mutants, respectively, and the polypeptide of pI 8.74 decreased in CM1707 (Fig. 5). The gene dosage effect in reciprocal F_1 and F_2 seeds of the crosses between Kinmaze and the glutelin mutants (Fig. 2) confirmed that the mutants were concerned with structural genes. Genetic analysis revealed that all of the α-2L mutants were controlled by co-dominant alleles, tentatively named as *glu4a*, *glu4b* and *glu4c*. The *glu4* locus was located on chromosome 1 linking with *eg* and *spl6* by crossover values of 24.2% and 17.2%, respectively (Fig. 4). Iida et al. (1997) mapped a *glu3* gene on chromosome 1 by RFLP. Similarly another *Glu1* gene, which was found in local rice cultivars, was also reported to be located on chromosome 1 (Nakamura et al. 1995). Deduced from their relative locations on chromosomal 1, it seems that *Glu1*, *glu3* and *glu4* are alleles or that their loci are linked closely to each other.

Iida et al. (1997) reported several kinds of low glutelin-content mutants and discussed the possibility of using the mutants for rice glutelin quantitative improvement. In this study, as seen in Figure 6, the *glu4* gene products of the pI 6.71/α-2 and pI 8.74/β-2 polypeptides were one of the major components of the glutelin acidic and basic subunits reflecting the expression of the gene in high amount. These results indicated that the *glu4* gene was one of the major glutelin genes. Therefore it is reasonable to expect using this gene for rice glutelin improvement by ordinary breeding and genetic engineering strategies. On the other hand, the α-2 subunit decreased whereas the other subunits increased in the *glu4a* and *glu4c* mutants. The decrease and increase of glutelin subunits reflected the alteration of glutelin components. These mutants would be useful for glutelin qualitative-improvement breeding.

Rice glutelin is estimated to be controlled by about ten genes per haploid genome (Okita et al. 1989; Takaiwa and Oono 1991). Glutelin was separated into thirteen acidic and eleven basic bands by IEF electrophoresis (Fig. 5), respectively. Therefore, the IEF bands of rice glutelin must correspond to the products of a system of structural genes, like that of zein in maize (Soave et al. 1978). Rice mature glutelin was derived from cleavage of the precursor; thus, one acidic polypeptide will certainly have one basic polypeptide to match it with. However, the pairing relations between the α polypeptide and β polypeptide have not been identified so far. The roughly equal numbers of acidic and basic polypeptides indicated the possibility to identify the pairing relationship between acidic and basic polypeptides. The acidic polypeptide of pI 6.71/α-2 and the basic polypeptide of pI 8.74/β-2 decreased simultaneously, and the two polypeptides did not separate at all in the F_2 generation of the cross between Kinmaze and CM1707 (data not shown). These results strongly suggested that pI 6.71/α-2 and pI 8.74/β-2 were the products of *glu4*, i.e. they were derived from the cleavage of the same precursor. The pairing relations of the remaining polypeptides are expected to be made clear by using relative mutants.

In CM1707, the glutelin polypeptides of pI 6.71/α-2 and pI 8.74/β-2 decreased simultaneously (Fig. 6). Although this might be caused by forming an abnormal proglutelin which was processed after translation, the results of Northern and RT-PCR analyses, together with the fact that the DNA sequences of the coding region of *glu4b* in CM1707 has no difference with that of *glu4* in Kinmaze (data not shown), excluded this possibility. The Northern and RT-PCR analyses revealed that the amount of mutated GluA-1 mRNA was tremendously decreased in CM1707 (Figs. 8A and 9) suggesting that CM1707 was a transcriptional mutation. The result that no difference was found in the amplification of PDI, which was used as a negative control, indicated that the differences of the PCR products of the mutated gene were not due to different amounts of total RNA.

To-date, more than eight glutelin cDNA clones have been isolated and sequenced and these clones have been classified into two subfamilies of GluA and GluB based on their nucleotide sequences. Six of them were deduced to be functional genes. However, the functions of these clones, i.e. their corresponding polypeptides observed in gel electrophoresis analyses, were not clear. The attempt of identifying glutelin gene function by comparing the N-terminal and internal amino-acid sequences with that deduced from the cDNAs (Hirano et al. 1991; Komatsu et al. 1993) were not satisfactory since the measured amino-acid sequences were fragmentary and the cDNA clones were of high homology. Some researchers tried to link the glutelin genes with cDNA clones by in situ hybridization (Suzuki et al. 1991) and by RFLP (Iida et al. 1997) using glutelin cDNA inserts as probes, yet the results lacked conviction because though these methods succeeded in distinguishing which subfamily the gene belongs to, they were not sensitive enough to identify which cDNA clone the gene corresponded to, due to the extremely high homology within the subfamily. In this study, the facts that N-terminal and internal amino-acid sequences of the pI 6.71/α-2 polypeptide in Kinmaze shared 100% homology with those deduced from the DNA sequences of GluA-1 suggested that the relative gene (*glu4*) belonged to GluA-1 (Fig. 7). Further, Northern-blotting analysis using three GluA cDNA clones and two GluB cDNA or genomic DNA clones as probes, revealed that *glu4* was a GluA-1 gene (Fig. 8A). RT-PCR analysis, using the GluA-1 specific nucleotide sequences as primers, confirmed the results of Northern analysis (Fig. 9). Though the GluA-1 and GluA-2 genes share 96% homology, the lower cross-hybridization of the GluA-1 probe to the GluA-2 and GluA-3 genes (Okita et al. 1989) permitted us to identify that CM1707 was a GluA-1 mutation. Summing up the results of biochemical and molecular analyses of CM1707, we unequivocally demonstrated that glutelin polypeptides of pI 6.71/α-2 and pI 8.74/β-2 were encoded by a GluA-1 gene. Similar rice mutants were considered to be useful materials for rice functional genomics studies.

There were several reports on the factors that affect the expression of the glutelin gene by analyzing the putative promoter of cDNA clones or by transgenic analysis of the cDNA clones, and some motifs such as GCN4, AACA, etc., have been identified (Zhao et al. 1994, 1995; Takaiwa et al. 1996; Yoshihara et al. 1996; Zheng and Murai 1997; Wu et al. 1998). The mutation mechanism of *glu4b* will be a useful component for studying the genetic regulatory mechanism of glutelin biosynthesis.

The pI 6.50 and pI 6.71 polypeptides disappeared from the *glu4b* and *glu4c* mutants. Accompanying the increase of the pI 6.50 polypeptide, a new pI 6.30 polypeptide emerged in the *glu4a* mutants (Fig. 5), and the two polypeptides did not separate in the F_2 generation of the cross between Kinmaze and the *glu4a* mutants (data not shown). These results suggested that pI 6.50 was also a product of *glu4,* and pI 6.30 that of *glu4a*. The different pIs and *Mrs* between them might be caused by post-translation modification (Hirano et al. 1991). It worth noting that though the acidic subunits changed significantly in the *glu4a* and *glu4c* mutants their basic subunits did not vary obviously. These results suggest that the mutation mechanism in the *glu4a* and *glu4c* mutants must be different from that of *glu4b*. The mutation mechanism of *glu4a* and *glu4c* are currently under investigation.

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