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Biochemical and molecular characterization of a rice glutelin allele for the GluA-1 gene

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Abstract The rice (*Oryza sativa* L.) mutant of *glu4a*, lacking the glutelin α-2 subunit while the α-1 subunit increased (α-1H/α-2L), was used in this study. Two-dimensional electrophoresis analysis revealed that the mutant lacked the polypeptide pI6.71/α-2 encoded by *glu4* while forming a new polypeptide of pI6.50/α-1. Experiments were conducted to identify the relationships between the mutated polypeptides of the mutant and to illustrate the mutation mechanism of the allele. Peptide mapping and amino-acid sequence analyses revealed that the newly formed *glu4a* encoded polypeptide pI6.50/α-1 of high homology with the deleted pI6.71/α-2 polypeptide which was encoded by *glu4* (GluA-1). The nucleotide sequence revealed that the iso-electric point variation of the pI6.50/α-1 polypeptide was caused by a point mutation with nucleotide replacement at the variable region of the gene. These results suggested the possibility of altering glutelin quality by using single gene mutation.

Keywords Rice glutelin · Mutant · Allele · Point mutation

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Introduction

The major seed-storage protein in rice is glutelin, which accounts for about 70% of total proteins on a weight basis (Ogawa et al. 1987; Li and Okita 1993). This character makes rice unique from other cereal crops except oat whose major storage proteins are prolamine. Rice glutelin is of high homology in structure to the 11S legume globulins (Luthe 1983; Zhao et al. 1983) containing a higher amount of the essential amino-acid lysine and is more easily digestible than prolamine (Tanaka et al. 1975; Ogawa et al. 1987; Resurreccion et al. 1993); therefore, it is one of the best plant proteins. Since glutelin is the predominate storage protein in rice seed, the seed quality and nutritional value are mainly determined by this protein.

Rice glutelin is synthesized as a 57 kDa precursor on the membrane-bound polysomes, and the precursor is processed proteolytically into a 40 kDa acidic (α) and a 20 kDa basic (β) subunit, respectively, within a protein storage vacuole called protein body II (PB II) (Yamagata et al. 1982; Furuta et al. 1986; Krishnan and Okita 1986; Yamagata and Tanaka 1986). The α and β subunits can be fractioned into three bands, namely α-1, α-2 and α-3, and β-1, β-2 and β-3 subunits, respectively, by SDS-PAGE analysis (Sarker et al. 1986; Kagawa et al. 1988). Each SDS-PAGE fractioned subunit is composed of at least two polypeptides, respectively, revealed by using iso-electric focusing (IEF) and two-dimensional electrophoresis (Wen and Luthe 1985; Qu et al. 2002), suggesting that the polypeptides correspond to the products of a group of structural genes.

Besides biochemical research, rice glutelin is intensively studied at the molecular level. To-date, more than eight glutelin cDNA and genomic DNA clones have been

isolated and sequenced (Takaiwa et al. 1987, 1991; Masumura et al. 1989; Okita et al. 1989; Takaiwa and Oono 1991). Molecular analyses of these genes revealed that there were three variable regions in the coding region of the acidic subunit which can lead to charge and size variation (Okita et al. 1989).

The rice genomic sequence has been completed and the functional genomic sequence is being intensively studied. Recently, we reported three kinds of allelic mutants lacking the glutelin α-2 subunit. The allele was temporally named as *glu4* which was located on chromosome 1 and corresponded to GluA-1 (Gt2) (Qu et al. 2002). The *glu4* gene encodes the polypeptides of pI6.71/α-2 and pI8.74/β-2. One of the allelic mutants (*glu4b*) was characterized by lacking these two polypeptides which enabled us to identify the gene function of *glu4*. Different from the *glu4b* mutant (CM1707), the *glu4a* mutant (EM278) had an increased α-1 subunit accompanied by a decreased α-2 subunit (α-1H/α-2L). The glutelin phenotypic diversity of the *glu4a* mutant indicated variation of the protein. Since any further studies concerning the molecular biology of rice glutelin will require a more complete understanding of its structure and composition, it is essential to extend the investigations including intra-locus variability and relationships among the polypeptides. This study deals with the biochemical characterization of the *glu4a* mutant, the relationships among the variant glutelin polypeptides and the molecular mutation mechanism of the alleles.

Materials and methods

Plant materials

The rice glutelin mutant EM278 (*glu4a*) lacking the α-2 subunit with the increased α-1 subunit (α-1H/α-2L), derived from the fertilized egg treated with N-methyl-N-nitrosourea (MNU) and its original cultivar of Kinmaze, were used as plant material.

Rice glutelin extraction and gel electrophoresis

Rice glutelin extraction, SDS-PAGE, IEF and two-dimensional electrophoresis (2-DE) analyses were conducted as previously described (Qu et al. 2002).

Peptide mapping analysis

Rice glutelin polypeptides were purified by two-dimensional electrophoresis. The appropriate band of the 2-DE was cut out with a razor blade, soaked with 125 mM Tris–HCl, pH 6.8, containing 0.1% (w/v) SDS and 1 mM of EDTA for 30 min. The gel slice was inserted into 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).

N-terminal and internal amino-acid sequence analysis

The N-terminal and internal amino-acid sequences were determined as previously described (Qu et al. 2002).

Isolation and nucleotide sequences of the glu4a gene

Rice genomic DNA was extracted from the leaf using cetyl trimethyl ammonium bromide (CTAB). The GluA-1 specific pairs of fragment were used as forward (5'-AAAAAGCAACAAAAG-CAAAAG-3') and reverse (5'-ACATAAGGATAAAGAGAGG-3') primers. Thermal cycling using Ex-*Taq* polymerase (Takara) was as follow: denaturing at 94 °C for 4 min, followed by 30 cycles of denaturing at 94 °C for 1 min 30 s, annealing at 58 °C for 2 min and extension at 72 °C for 2 min 30 s. The PCR products were subcloned into pT7 blue vector (Novagen) using a ligation high kit (Toyobo). The nucleotide sequences were determined by using the ABI 310 Genetic Analyzer (Applied Biosystems). The cDNA sequence was determined by comparing that reported by Okita et al. (1989).

Results

The *glu4a* mutant contained variant glutelin polypeptides

SDS-PAGE analysis of the α-1H/α-2L mutant of EM278 (*glu4a*) showed that the α-1 subunit increased while the α-2 subunit disappeared (Fig. 1A). IEF analysis of the mutant revealed that an acidic subunit with a 6.50 pI value (pI6.50) increased greatly in the mutant accompanied by the pI6.71 subunit which decreased (Fig. 1B) (Qu et al. 2002). The two subunits differed at the 0.2 pI unit. To ascertain whether the increased band in IEF and in SDS-PAGE was caused by forming a new polypeptide or by increasing the related band of the glutelins were analyzed by two-dimensional electrophoresis (2-DE). 2-DE analysis revealed that the pI6.71/α-2 polypeptide decreased tremendously in the mutant compared to that in Kinmaze (Fig. 2). In EM278, the pI6.50 band increased and shifted from the position of α-2 in Kinmaze to the α-1

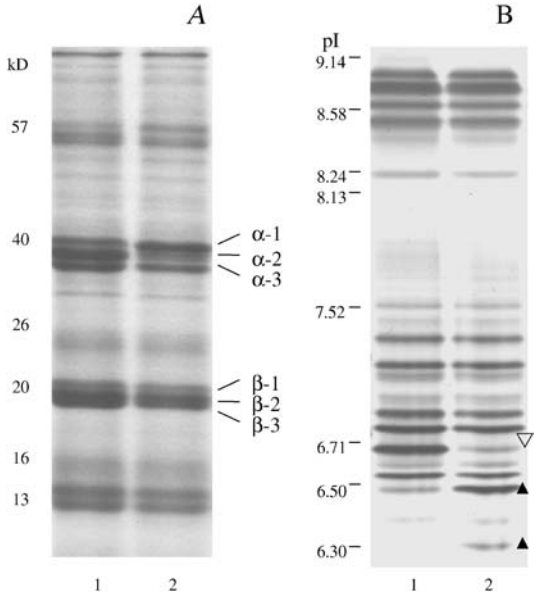

Fig. 1 Analyses of glutelin composition from Kinmaze (*glu4*) and EM278 (*glu4a*) by SDS-PAGE (**A**) and IEF (**B**). The *black arrowhead* and *white arrowhead* indicate increased and decreased polypeptides, respectively. *Lane 1* Kinmaze; *lane 2* EM278

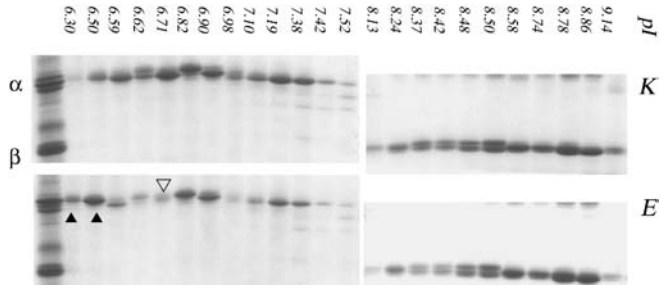

Fig. 2 Two-dimensional electrophoresis analysis of glutelin fractions from Kinmaze (K, *glu4*) and EM278 (E, *glu4a*). The *black arrowheads* and *white arrowhead* indicate new polypeptides and a decreased polypeptide, respectively

position in the mutant. In addition, the increased pI6.30 band of EM278 was also confirmed to be a component of the α-1 subunit. These results indicated that α-1H was caused by forming the new polypeptides of pI6.50/α-1 and pI6.30/α-1.

The mutated polypeptides are homologous

In EM278, the polypeptide of pI6.71/α-2 decreased, while the new polypeptide of pI6.50/α-1 was formed. It was assumed that the two polypeptides might have some relationship since they were controlled by co-dominant alleles (Qu et al. 2002). To reveal the relations between them, the polypeptides were treated with the *S. aureus* V8 protease (this protease cleaves at the COOH-terminal side of the aspartic and glutamic acid residues) and the digestions were fractionated by tricine-SDS-PAGE. When the polypeptides were treated with the protease, nine peptide bands were obtained from pI6.50/α-1 and pI6.71/α-2, respectively, and the band patterns were the same (Fig. 3). The fact that the peptide band patterns of pI6.50/α-1 and pI6.71/α-2 were identical but different from other polypeptides (data not shown), suggested that these two polypeptides were homogeneous.

After de-blocking with pyroglutamate aminopeptidase the glutelin acid polypeptides were subjected to a gas-phase protein sequencer to determine the N-terminal amino-acid sequence of the polypeptides. The amino-acid sequences of pI6.71/α-2 of Kinmaze and pI6.50/α-1 of EM278 were determined from the second residue. The N-terminal amino-acid 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 no difference between the two polypeptides (Fig. 4).

To illustrate the relationship between the two polypeptides based on sequence information, it was desirable to elucidate the amino-acid sequence not only of the N-terminal region but of the internal region of the protein as well. The internal amino-acid sequences of the two polypeptides were also determined (Fig. 4). The results showed that the ten residues of the internal amino-acid

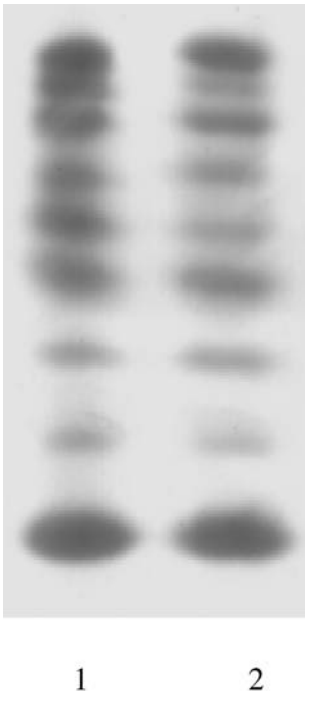

Fig. 3 Peptide mapping band patterns of the mutated polypeptide obtained after digestion with *S. aureus* V8 protease. *Lane 1*, pI6.71/α-2 of Kinmaze; *lane 2*, pI6.50/α-1 of EM278

	10	120
Glutelin A-1	<QQLLGQSTSQ------SQSQSQKFKD---	
pI6.71/α-2	<QQLLGQSTSQ------SQSQSQKFKD---	
pI6.50/α-1	<QQLLGQSTSQ------SQSQSQKFKD---	

Fig. 4 Amino-acid sequences of mutated glutelin polypeptides. Glutelin A-1: the sequences deduced from the nucleotide sequence of the GluA-1 cDNA clone. < Q represents pyroglutamic acid

sequence of the two polypeptides were 100% homologous to each other.

The result that both N-terminal and internal amino-acid sequences of the two polypeptides of pI6.50/α-1 and pI6.71/α-2 were identical to each other, suggested that they might be derived from one ancestor. In other words, the newly formed polypeptide of pI6.50/α-1 might derive from that of pI6.71/α-2.

The pI variant was caused by nucleotide replacement in the variable region of the *glu4* gene

Since the *glu4* corresponded to GluA-1 (Gt2), the Gt2 specific fragments were used as primers to amplify the *glu4* and *glu4a* gene by PCR. The amplified genes were subcloned into the pT7 blue vector and their nucleotide sequences were determined. By determining the nucleotide sequences of the *glu4* and *glu4a* genes, it was possible to compare directly between them. Despite the high conservation between the genes, some differences

Fig. 5 cDNA sequence of *glu4* and *glu4a*. Nucleotide replacements are indicated, whereas an asterisk indicates no change. Theinitiation codon and stop codon are *bold printed*

```
glu4   ATGGCATCCATAAATCGCCCCATAGTTTTCTTCACAGTTTGCTTGTTCCTCTTGTGCAATGGCTCTCTAGCCCAGCAGCTATTAGGCCAG 90
glu4a ********************************************************************************************

glu4   AGCACTAGTCAATGGCAGAGTTCTCGTCGTGGAAGTCCAAGAGAATGCAGGTTCGATAGGTTGCAAGCATTTGAGCCAATTCGGAGTGTG 180
glu4a ********************************************************************************************

glu4   AGGTCTCAAGCTGGCACAACTGAGTTCTTCGATGTCTCTAATGAGCAATTTCAATGTACCGGAGTATCTGTTGTCCGTCGAGTTATTGAA 270
glu4a ********************************************************************************************

glu4   CCTAGAGGCCTTCTACTACCCCATTACACTAATGGTGCATCTCTAGTATATATCATCCAAGGGAGAGGTATAACAGGGCCAACTTTCCCA 360
glu4a ********************************************************************************************

glu4   GGCTGTCCTGAGTCCTACCAACAACAGTTCCAACAATCAGGCCAAGCCCAATTGACCGAAAGTCAAAGCCAAAGTCAAAAGTTCAAGGAT 450
glu4a ********************************************************************************************

glu4   GAACATCAAAAGATCCACCGTTTCAGACAAGGAGATGTAATTGCATTGCCTGCTGGTGTAGCTCATTGGTGCTACAATGATGGTGAAGTG 540
glu4a ********************************************************************************************

glu4   CCAGTTGTTGCCATATATGTCACTGATCTCAACAACGGTGCTAATCAACTTGACCCTAGGCAAAGGGATTTCTTGTTAGCTGGAAATAAG 630
glu4a ********************************************************************************************

glu4   AGAAACCCTCAAGCATACAGGCGTGAGGTTGAGGAGCGGTCACAGAACATATTTAGTGGCTTTAGCACTGAACTACTTAGCGAGGCTCTT 720
glu4a ********************************************************************************************

glu4   GGCGTAAGCAGCCAAGTGGCAAGGCAGCTCCAATGTCAAAATGACCAAAGAGGAGAAATTGTCCGTGTCGAACACGGGCTCAGTTTGCTG 810
glu4a ********************************************************************************************

glu4   CAGCCATATGCATCATTGCAGGAGCAGGAACAAGGACAAGTGCAATCAAGAGAGCGTTATCAAGAAGGACAATATCAGCAAAGTCAATAT 900
glu4a ****************C***************************************************A**********************

glu4   GGAAGTGGCTGCTCTAACGGTTTGGATGAGACCTTTTGCACCCTGAGGGTAAGGCAAAACATCGATAATCCTAACCGTGCTGATACATAC 990
glu4a ********************************************************************************************

glu4   AATCCAAGAGCTGGAAGGGTTACAAATCTCAACACCCAGAATTTCCCCATTCTTAGTCTTGTACAGATGAGTGCAGTCAAAGTAAATCTA 1080
glu4a ********************************************************************************************

glu4   TACCAGAATGCACTCCTTTCACCATTTTGGAACATCAACGCTCACAGCGTCGTGTATATTACTCAAGGCCGTGCCCGGGTTCAAGTTGTC 1170
glu4a ********************************************************************************************

glu4   AACAACAATGGAAAGACAGTGTTCAACGGCGAGCTTCGCCGCGGACAGCTGCTTATTATACCACAACACTATGCAGTTGTAAAGAAGGCA 1260
glu4a ********************************************************************************************

glu4   CAAAGAGAAGGATGTGCTTACATTGCATTCAAGACCAATCCTAACTCTATGGTAAGCCACATTGCAGGAAAGAGTTCCATCTTCCGTGCT 1350
glu4a ********************************************************************************************

glu4   CTCCCAAATGATGTTCTAGCAAATGCATATCGCATCTCAAGAGAAGAGGCTCAGAGGCTCAAGCATAATAGAGGAGATGAGTTCGGTGCA 1440
glu4a ********************************************************************************************

glu4   TTCACTCCAATCCAATACAAGAGCTACCAAGACGTTTATAATGCGGCAGAATCCTCTTAGGTCGGCTTGCGGATAAAGAATAACTAAATA 1530
glu4a ********************************************************************************************

glu4   AATAAATTGCAAGCAATTGTTTTGCTGCTATGTACTGTCCAGTCTTACGAC
glu4a **********************************************
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Fig. 6 Deduced primary sequence of the polypeptides encoded by *glu4* and *glu4a*. Amino-acid replacements are indicated, whereas an asterisk indicates no change

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glu4   MASINRPIVFFTVCLFLLCNGSLAQQLLGQSTSQWQSSRRGSPRECRFDRLQAFEPIRSVRSQAGTTEFFDVSNEQFQCTGVSVVRRVIE  90
glu4a ********************************************************************************************

glu4   PRGLLLPHYTNGASLVYIIQGRGITGPTFPGCPESYQQQFQQSGQAQLTESQSQSQKFKDEHQKIHRFRQGDVIALPAGVAHWCYNDGEV 180
glu4a ********************************************************************************************

glu4   PVVAIYVTDLNNGANQLDPRQRDFLLAGNKRNPQAYRREVEERSQNIFSGFSTELLSEALGVSSQVARQLQCQNDQRGEIVRVEHGLSLL 270
glu4a ********************************************************************************************

glu4   QPYASLQEQEQGQVQSRERYQEGQYQQSQYGSGCSNGLDETFCTLRVRQNIDNPNRADTYNPRAGRVTNLNTQNFPILSLVQMSAVKVNL 361
glu4a *****S***************E**************************************************************************

glu4   YQNALLSPFWNINAHSVVYITQGRARVQVVNNNGKTVFNGELRRGQLLIIPQHYAVVKKAQREGCAYIAFKTNPNSMVSHIAGKSSIFRA 450
glu4a ********************************************************************************************

glu4   LPNDVLANAYRISREEAQRLKHNRGDEFGAFTPIQYKSYQDVYNAAESS
glu4a **********************************************
```

were observed in the coding and non-coding regions of the genes. The most conspicuous differences were point mutations T-to-C at position 827 (start from the initiator ATG codon) and G-to-A at position 878 (Fig. 5) in the third exon (Okita et al. 1989), leading to the amino-acid changed from leucine to serine and from glycine to glutamic acid, respectively (Fig. 6). Though the former amino-acid replacement did not cause charge variation, the later replacement reduced the charge of the mutated polypeptide since the neutral amino acid of glycine was placed by an acidic amino-acid of glutamic acid. This result revealed the reason why the variant glutelin polypeptide of pI6.50/α-1, the product of *glu4a*, decreased about 0.2 pI units compared to that of the wild-type of pI6.71/α-2. The charge-reduced nucleotide replacement occurred in the hyper-variable region (HVR) (Okita et al. 1989) of the gene, and the other mutation

occurred just before the HVR. No variations were found in the region encoding the basic chain.

Discussion

Rice glutelin is synthesized in the rough endoplasmic reticulum (rER) as a 57-kDa precursor, and the precursor is processed proteolytically to an acidic subunit and a basic subunit after removal of the signal peptide within PB II (Yamagata et al. 1982; Krishnan and Okita 1986; Sarker et al. 1986; Yamagata and Tanaka 1986). The family of glutelin genes in rice has been divided into two subfamilies, GluA and GluB, based on properties of the subunits they encoded. The genes in GluA, which includes GluA-1, GluA-2, GluA-3 and GluA-4, have nucleotide sequences that are 73–96% homologous to one another (Takaiwa et al. 1987, 1991; Okita et al. 1989). The nucleotide sequences for members of the GluB subfamily, which includes GluB-1, GluB-2, GluB-3 and GluB-4, are more than 80% identical with one another, but are 60–65% homologous with those in the GluA subfamily (Masumura et al. 1989; Takaiwa et al. 1991). GluA-4 and GluB-3 were considered to be pseudo-genes since there existed a stop codon in the coding regions. The others were deduced to be functional genes. To-date, only the function of the GluA-1 gene, which corresponded to *glu4*, has been made clear for encoding the polypeptides of pI6.71/α-2 and pI8.74/β-2 (Qu et al. 2002).

The *glu4* locus, which was located on chromosome 1, contained at least three alleles encoding different glutelin polypeptides. One of them is *glu4a* (EM278). Two-dimensional electrophoresis analysis revealed that the *glu4a* (α-1H/α-2L) mutant lacked the polypeptide of pI6.71/α-2, while it formed a new polypeptide of pI6.50/α-1 (Fig. 2). Peptide maps of the variant pI6.50/α-1 polypeptide and the pI6.71/α-2 polypeptide, the wild-type, were the same (Fig. 3), and their amino-acid sequences were identical at both the N-terminus and in the interior (Fig. 4). These results suggested that the newly formed polypeptide of pI6.50/α-1 in the mutant was derived from the pI6.71/α-2 polypeptide.

The rice glutelin primary sequence had significant homology with that of the legume 11S globulin (Zhao et al. 1983; Higuchi and Fukazawa 1987; Takaiwa et al. 1987). Comparing the glutelin primary sequence with that of soybean glycinin and pea legumin, Higuchi and Fukazawa (1987) demonstrated that the divergent domain of the glutelin acidic subunit corresponded to the so-called hyper-variable region (HVR) in the legume 11S storage protein (Argos et al. 1985), which enabled the protein to tolerate a large insertion of variable size. Okita et al. (1989) reported that in the rice glutelin acidic subunit there were two variable regions besides the HVR, that appeared to tolerate peptide changes leading to the variation in overall size and net charge. *Glu4a* was expressed normally in the mutant, and the N-terminal and internal amino-acid sequences of its product were identitical with that of *glu4* (Fig. 4); however, their pIs and sizes were different (Figs. 1, 2) reflecting the nucleotide sequence differences in their coding regions. Biochemical analysis revealed that the products of *glu4a* and *glu4* (the polypeptides of pI6.50/α-1 and pI6.71/α-2) differed both in charge and size (Fig. 2). Nucleotide sequence analysis revealed that the gene difference was caused by nucleotide replacements at the variable region (Fig. 5). The amino-acids of leucine and glycine in the pI6.71/α-2 polypeptide were replaced by serine and acedic-amino acid of glutamic acid, respectively, leading to the formation of the new polypeptide pI6.50/α-1 (Fig. 6). The later amino-acid replacement illustrated the decreased charge of the 0.2 pI unit in the pI6.50/α-1 polypeptide. The size increase of the new polypeptide might be caused by the post-translational process such as glycosilation, though we failed to identify glycosilation in the variant polypeptide due to the insolubility of the glutelin in the buffer with a low urea concentration. The size increase of the new polypeptide might also be caused by the amino-acid replacements which reduced the binding ability of the polypeptide with SDS leading to the lower migration of the polypeptide with the appearance of increased size.

A number of soybean seed-protein mutants with changes in either their charge or size have been reported (Orf and Hymowitz 1979; Hildebrand and Hymowitz 1980; Davies et al. 1985). However, the precise molecular events responsible for these changes remain unclear. In this paper we demonstrate that the newly formed glutelin polypeptide of pI6.50/α-1, with a changed charge and size in the *glu4a* mutant (EM278), was caused by two point mutations involving nucleotide replacement compared to the wild counterpart of *glu4*. This result might be informative for the evolution of rice glutelin genes since similar α-1H/α-2L mutants were also found in local cultivars from south Asia (Satoh et al., unpublished data). It worth noting that different polypeptides might be the products of one gene by the accumulation of nucleotide replacements in the non-conserved regions of the gene, indicating the feasibility for improving nutritional qualities by altering these regions by genetic modification.

In the *glu4a* mutant, in addition to pI6.50/α-1, the polypeptide of pI6.30/α-1 was newly formed. The polypeptides of pI6.30/α-1 and pI6.50/α-1 did not separate in the F_2 generation of the cross between EM278 and Kinmaze (data not shown). Since the polypeptides of pI6.30/α-1 and pI6.50/α-1 always inherit together, they would be single-gene products formed by post-translational modification, such as glycosilation (Hirano et al. 1991), resulting in a different charge and size.

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