

DIVERSITY OF GLUTELIN ACIDIC SUBUNIT POLYPEPTIDES IN RICE CULTIVARS COLLECTED FROM NORTHERN VIETNAM

Tran Thi Thu Hoai^{1,2}, Tran Danh Suu², and Toshihiro Kumamaru¹

1. Institute of Genetic Resources, Faculty of Agriculture, Kyushu University

2. Plant Resources Center, Vietnamese Academy of Agricultural Science (VAAS)

Abstract

The diversity of glutelin acidic polypeptides in rice cultivars collected from northern Vietnam was characterized via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) electrophoresis. Glutelin acidic subunits were separated into 4 bands by molecular mass, as α -1 (39 kDa), α -2 (38 kDa), α -3 (37.5 or 37 kDa), and α -4 (34 or 33 kDa). One hundred and eighty-five Vietnamese rice cultivars were divided into 3 types, based on differences in staining intensity and the molecular size of the α -3 and α -4 polypeptides derived from SDS-PAGE analysis. Wide variation was also observed in the isoelectric point (pI) staining intensity, in addition to the absence/presence of pI bands detected via IEF analysis. A total of 16 pI bands, ranging from pI 6.30 to pI 7.52, were identified in the various local rice cultivars. The maximum and minimum of IEF bands detected were 14 and 10, respectively. The genetic variability index (H') ranged from 0.280 to 0.820, which confirms that local rice cultivars from northern Vietnam have diverse glutelin seed storage units.

Key words: electrophoresis, seed storage glutelin, diversity, rice germplasm

Introduction

Cereals are an important source of dietary protein for humans. Rice is unquestionably a superior source of energy among existing cereal crops. Rice is the most important food crop worldwide, providing over 21% of the calorific needs of the world's population in South East Asia. (Fitzgerald et al. 2008).

Seed storage proteins do not contain enzymes, and have the sole purpose of providing the proteins (nitrogen and sulfur source) required for germination and the establishment of a new plant (Mandal and Mandal 2000). Seed storage proteins are classified based on their solubility in water (albumins), dilute saline (globulin), aqueous alcohol (prolamin), and dilute acid or alkali (glutelin) (Osborn 1924). Prolamin and glutelin are the major seed storage proteins found in rice. Prolamin is deposited in protein body I, which is derived from the endoplasmic reticulum (ER). In comparison, glutelin is deposited in protein body II, which is derived from the protein storage vacuole (PSV) (Tanaka et al. 1980; Ogawa et al. 1987). Glutelin, which accounts for 80% of total rice storage proteins, is easily digested. In contrast, it is difficult

to digest prolamin, which accounts for about 20% of total rice storage proteins.

There has been extensive effort to improve the protein content of rice grains. Several seed storage protein mutants have been artificially generated through the use of *N*-methyl-*N*-nitrosourea (MNU) mutagenesis (Satoh and Omura 1981; Kumamaru et al. 1988). It is important to analyze these mutants to understand the synthesis, processing, and deposition of seed storage proteins in rice. In addition to artificial mutants, spontaneous seed storage protein mutants have also been found in the local rice cultivars of some countries (Jahan et al. 2001; Aung et al. 2003). Characterization of seed storage protein in rice germplasm, therefore, plays a critical role in developing the novel genetic resources for rice quality improvement.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a valuable tool for characterizing variation in the storage proteins of different rice cultivars. Proteins may be separated by size under denaturing conditions; therefore, this method also facilitates the estimation of the relative molecular mass of individual polypeptides (Hilu and Essen 1988). While SDS-PAGE separates proteins based on their molecular masses, iso-electric focusing (IEF) separates proteins based on the isoelectric point. Moreover, each subunit fractionated by SDS-PAGE is composed of at least 2 polypeptides, which are isolated through IEF electrophoresis (Wen and Luthe 1985; Uemura et al. 1996; Qu et al. 2002). Many studies have evaluated the genetic diversity of seed storage proteins from local crop cultivars using by SDS-PAGE. Examples include *Brassica* (Rahman and Hirata 2004), triticale (Igrejas et al. 1999), foxtail millet (Kumarn et al. 1998), cereal (Damania et al. 1983; Gorinstein et al. 1999; Alvarez et al. 2006; Lerner et al. 2009), *Arachis* (Bertozzo and Valls 2001), and alfalfa bean (Zivkovi'c et al. 2012). Some studies have demonstrated that the glutelin profile is highly varied in local rice cultivars sampled from Madagascar, Myanmar, Bangladesh and Pakistan (Satoh et al. 1990; Aung et al. 2003; Jahan et al. 2005; Siddiqui et al. 2003, 2010). Studies focusing on variation in the glutelin profile might provide valuable information for collecting and conserving genetic resources, in addition to the broadening the genetic material available for seed quality enhancement programs.

Previous studies have reported high genetic diversity in *O. sativa* cultivars from China, Thailand, Laos, Bangladesh, Myanmar and Vietnam (Oka 1988; Jahan et al. 2001; Aung et al. 2003; Fukuoka et al. 2003, 2006). As reported by Chang (1976), the belt of primary genetic diversity extends from the Ganges Plains below the eastern foothills of the Himalayas, through upper Burma, northern Thailand, Laos, and northern Vietnam, to the southwestern and southern parts of China. This phenomenon is supported by the existence of high genetic diversity of larger numbers of local rice cultivars in the Northern Vietnam (Trinh et al. 1993; Okuno et al. 1996; Fukuoka et al. 2003, 2006).

In this study, we examined the diversity of glutelin in local rice cultivars from northern Vietnam using SDS-PAGE and IEF analyses. Information on the geographic distribution of glutelin and genetic variation of rice cultivars obtained in this study is anticipated to (1) provide baseline information on the

genetic diversity of rice, (2) contribute new information about the synthesis, processing, and deposition of seed storage proteins in rice, and (3) facilitate the conservation of unique rice cultivars, as well as provide new genetic material to for rice quality enhancement programs.

Materials and methods

Plant materials

One hundred and eighty-five rice cultivars, *Oryza sativa*, were collected from 8 provinces in the northern mountainous areas of Vietnam. These cultivars are now preserved in National seed genebank of plant resources center in Vietnam. The Japanese rice cultivar ‘Kinmaze’ was *japonica* rice while the cultivar ‘IR36’ developed by IRRI was *indica* rice, were used as controls.

Protein extraction

Total protein was extracted from 1 seed of each cultivar. Each grain was ground using pliers, and powdered seed from each sample was placed in a 1.5-ml micro test tube containing 700 μ l SDS-PAGE sample buffer solution, 0.125 M Tris HCl (pH 6.8), 4 M urea, 4% SDS, and 5% 2-mercaptoethanol. Then, the samples were vibrated on the shaker overnight, at room temperature. The protein in resultant supernatant was used for SDS-PAGE, after being centrifuged at 14,000 rpm for 10 min at 4°C.

Glutelin extraction

A single mature seed from each cultivar was prepared for each sample. The crushed seed and albumin-globulin extraction buffer (10 ml Tris HCl solution, of pH 6.8, containing 0.5 M NaCl) were placed into a 1.5-ml micro test tube. After washing the mixture with albumin-globulin extraction buffer, the supernatant was neutralized with 1 M NaOH, adjusted with 1% lactic acid, and then centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was discarded, and the precipitate was dissolved in 200 μ l 4M SDS-PAGE sample buffer, of which 10 μ l was removed for SDS-PAGE.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was conducted by using the discontinuous buffer system of Laemmli (1970) on slab gel containing a linear concentration gradient of 15–25% acrylamide and 0.05–0.67% bisacrylamide. After electrophoresis, the gel was stained in 50% methanol, 7% acetic acid, and 0.1% Coomassie brilliant blue (CBB) R-250.

Isoelectric focusing (IEF)

IEF gel was prepared according to Uemura et al. (1996), composed of 8.5 M urea, 30% acrylamide, 1,5%

bis acrylamide, 20% Nonidet P 40, and 2% ampholine with a pH range of (3.5–10); (6–8); (8–10.5) = 1:1:1. The glutelin extract from the rice seed was dissolved in O'Farrell solution containing 8.5 M urea, 2% Nonidet P-40, and 5% 2-mercaptoethanol (O'Farrell 1975), and was then used for IEF analysis. Electrofocusing was conducted at 300 V for 1 h, 900 V for 1 h and 20 min, 1,200 V for 1 h, and 1700 V for 1 h. After electrofocusing, the gel was fixed in 15% trichloroacetic acid (TCA) for 15 min. It was then placed in destaining solution for 12 h, and then placed in a staining solution containing 50% ethanol, 10% acetic acid, and 0.15% comassie brilliant blue R-250 for 20 min. The gel was then transferred to a, until the background was clear, and was then washed with distilled water.

Results

Variation in glutelin profiles based on SDS-PAGE analysis

Rice glutelin was composed of acidic and basic subunits. SDS-PAGE analysis further separated the glutelin acidic subunit into 4 bands of different molecular size; specifically, α -1 (39 kDa), α -2 (38 kDa), α -3 (37.5 or 37 kDa), and α -4 (34 or 33 kDa). The glutelin basic subunits were separated into 3 bands; specifically, β -1 (23 kDa), β -2 (22.5 kDa), and β -3 (22 kDa). 'Kinmaze' was differentiated from 'IR36' based on differences in the molecular size of α -3 and α -4. The molecular size of 'Kinmaze' α -3 (37 kDa) was smaller compared to that of 'IR36' (37.5 kDa). The molecular size of 'Kinmaze' α -4 (34 kDa) was larger compared to that of 'IR36' (33 kDa) (Uemura et al. 1996). On the basis of differences in the staining intensity and molecular size of glutelin acidic subunits, the Vietnamese local rice cultivars were divided into 3 types. Type I was described as an 'IR36' type, while Type II was described as 'Kinmaze' type. Type III contained the α -3' band and the 'IR36' type. The number of cultivars represented by Types I, II, and III were 59 (32%), 124 (67%), and 2 (1%), respectively.

Variation in the glutelin profiles of Vietnamese rice cultivars based on IEF analysis

The IEF analysis provides further information about the variation of glutelin polypeptides based on their isoelectric points (pI). A major difference between 'Kinmaze' and 'IR36' was observed using the IEF glutelin acidic profile. The glutelin acidic subunit of 'Kinmaze' and 'IR36' were separated into 14 polypeptide bands. 'Kinmaze' contained high intensities of pI 7.19, pI 6.98, and pI 6.71 ('Kinmaze'-specific bands), while pI 7.15, pI 6.80, and pI 6.59 were missing ('IR36'-specific bands), which differentiated it from 'IR36'. High variation in IEF glutelin acidic polypeptides was observed among the local Vietnamese rice cultivars, with the pI ranging from 6.30 to 7.52. Varieties belonging to SDS-PAGE types I, III, and II correspond to the IEF types shown in lanes 3–10, 11, and 12–16, respectively. In addition to the 14 bands found in 'Kinmaze' and 'IR36', 4 IEF bands (pI 7.40, pI 6.30, pI 7.17, and pI 7.52 in lanes 3, 4, 9, and 11, respectively) were detected in local Vietnamese rice cultivars. Lanes 3 to 10 (SDS-PAGE

type I)-were characterized by the presence of pI 6.59 and pI 6.80, and the low staining intensity of pI 6.71. In contrast, lanes 12–16 (SDS-PAGE type II- were characterized by the absence of pI 6.59 and pI 6.80, along with a high intensity of pI 6.71. A high intensity of pI 7.52 and low intensity of pI 7.19 was found in lane 11 of SDS-PAGE type III, whereas the pI patterns of the 6.59, 6.71, and 6.80 bands matched those of SDS-PAGE type I. There was wide variation in the pI of SDS-PAGE type I cultivars, in addition to a wide variation in the staining intensity of the 4 IEF bands (namely, pI 6.50, pI 6.55, pI 6.90, and pI 6.98). A new pI band (7.17) was only detected in SDS-PAGE type I (lane 9), while the 7.40 pI band was recorded in both SDS-PAGE types I (lanes 3, 5, and 6) and II (lane 13). The 7.52 pI band was observed in types III and II of SDS-PAGE (lanes 11 and 13, respectively). Among the SDS-PAGE type II cultivars, a low staining intensity of pI 6.98 and high staining intensity of pI 7.38 was detected in lane 16.

The number of IEF bands ranged from 10 to 14. The differences in staining intensities of glutelin acidic IEF bands were not similar among local rice cultivars, and were recorded as high, present (normal type), and low intensity. This shows the variation of IEF glutelin acidic bands recorded in local rice cultivars. Variations were detected in 14 of the 16 IEF bands (excluding pI 6.62 and 6.82). Two IEF bands, pIs 6.50 and 6.55, exhibited the highest glutelin acidic band diversity, because they contained all of the variation.

Glutelin variation and geographical distribution

The geographical distribution of glutelin variation in local Vietnamese rice cultivars based on SDS-PAGE is shown. Geographical differences were found in a number of cultivars among provinces. In this research, we used the genetic variability index (H') of Shannon and Weaver (Shannon and Weaver 1949) as: $H' = -\sum pI \ln pI$, where pI is the relative frequency of each glutelin type. The genetic variability index (H') of the 4 northeast provinces (composed of Bac Thai, Cao Bang, Tuyen Quang, and Lang Son) was 0.610, 0.820, 0.280, and 0.660, respectively. The Cao Bang province contained all variations of the SDS-PAGE glutelin type, and showed the highest level of genetic variability ($H' = 0.820$). The genetic variability index of the 4 northwest provinces (composed of Lao Cai, Son La, Dien Bien, and Lai Chau) was 0.70, 0.690, 0.685, and 0.630, respectively. Thus, rice from the northeast had a higher glutelin SDS-PAGE diversity pattern compared to that from the northwest.

The geographical distribution of the glutelin acidic subunit in local Vietnamese rice cultivars is shown. Rice from both the northeast and northwest regions exhibited wide variation in relative band intensity, as well as in band presence or absence among local rice cultivars. Rice from the northeast region had higher IEF band pattern diversity compared to that from the northwest. The newly detected pI bands 7.40 and 7.52 were distributed in both regions. Of interest, while the new pI band 7.17 was only found in the northwest region, the new pI band 6.30 was only present in the northeast region. The pI band 6.50 of northwest rice and the pI band 6.55 of northeast showed the highest variation, in terms of high or low

relative band intensity and band presence or absence. Thus, rice from the northwest and northeast regions had high diversity in the IEF profiles of the glutelin acidic subunit.

Discussion

The characterization of seed storage protein in local rice cultivars (Minakuchi et al. 1994; Satoh et al. 1990, 1995; Jahan et al. 2001, 2005; Aung et al. 2003; Siddiqui et al. 2003, 2010) and in artificial mutants (Satoh and Omura 1981; Kumamaru et al. 1987, 1988; Kagawa et al. 1988; Satoh et al. 1997) has been carried out. However, there was a limited in formation of seed storage protein from Vietnamese local rice cultivars was reported. In this study, wide variation in glutelin subunits was observed in local Vietnamese rice cultivars through SDS-PAGE analysis. Wide diversity in relative band staining intensity, as well as molecular size, was found for 4 SDS-PAGE bands of the glutelin acidic subunit. A significant difference in the staining intensity and molecular size of the α -3 and α -4 bands was recorded among local rice cultivars, with the isolation of 3 types; specifically, type I ('IR36' type), type II ('Kinmaze' type), and type III (two α -3 bands). Because the relative band intensity differed remarkably among the cultivars of each type, it is suggested that the α -3 and α -4 bands might be encoded by different genes. In addition to variation in the migration of the α -3 and α -4 bands, 2 α -3 polypeptide bands (type III) were observed for 2 local rice cultivars. The SDS-PAGE pattern of type III had a similar α -3 and α -4 band molecular size to that of type I. Moreover, the pI band 6.59 and pI band 6.80 were present in type III, indicating that type III might have from the type I, as a result of selection and maintenance processes of certain local group peoples in this region. Similar spontaneous glutelin mutations have also been documented in Madagascar, Asia, Myanmar, Bangladesh, Pakistan rice (Satoh et al. 1990; Uemura et al. 1996; Aung et al. 2003; Jahan et al. 2005; Siddiqui et al. 2003, 2010). It may be concluded that the Asian rice germplasm is highly diverse due to the spontaneous mutations of glutelin. The spontaneous glutelin mutations that are found in local rice cultivars might help improve our understanding about the synthesis, regulation, and deposition of seed storage glutelin in rice endosperm.

Uemura et al. (1996) found that the glutelin α subunit contains 11 distinct IEF bands. IEF analysis showed that local rice cultivars had a wide variation in relative band intensity, pI, and the number of bands. Variation in the staining intensity of the IEF profile for the glutelin acidic subunit was observed in 14 of the 16 IEF bands. Two of the 16 glutelin acidic subunit IEF bands exhibited high diversity in the staining intensity and the absence or presence of pI. Previous studies have also documented the diversity in the intensity and the absence/presence of the IEF bands (Aung et al 2003; Jahan et al. 2005; Siddiqui et al. 2003). However, the correlation between the glutelin subunit components and their encoding genes has yet to be established. Research about the diversity of glutelin polypeptide diversity in rice germplasm is vital to identify the correlation between glutelin polypeptides and their encoding genes. The analysis on the geographical distribution of the glutelin acidic subunit based on SDS-PAGE demonstrated that rice from

north Vietnam contains a high diversity of glutelin seed storages. Fourteen of the 16 detected IEF bands exhibited a similar geographical distribution, with differences in just 2 IEF bands from northwest and northeast regions being observed. Northern Vietnam falls within the area of maximum rice diversity (Chang 1976). Thus, the wide variation of the glutelin subunit documented in this study supports that priority should be given toward collecting and conserving the genetic rice resources of this region.

Seed storage proteins in rice are encoded by families of polymorphism genes (Kataoka 1978; Kambayashi et al. 1984; Okita et al. 1989). As reported by Okita et al. (1989) and Takaiwa et al. (1991), glutelin is encoded by at least 10 copies of each haploid genome. Rice glutelin contains 2 nutritionally different subfamilies A and B (Tanaka et al. 2004). Qu et al. (2003) reported that the mutant lacked the polypeptide pI6.71/ α -2 encoded by *GLU4* gene, while forming a new polypeptide (pI6.50/ α -1), which was encoded by the *GLU4a* gene. By using IEF and two-dimensional electrophoresis analysis, Wen and Luthe (1985) and Qu et al. (2002) found that each SDS-PAGE fractioned subunit is composed of at least 2 polypeptides. The 2 research groups suggested that the polypeptide corresponds to the product of structure genes. In this study, both the SDS-PAGE and IEF patterns of the glutelin subunit were found to exhibit wide variation. Thus, this result is consistent with the glutelin subunits of rice reported by Jahan et al. (2005) and Aung et al. (2003) in Bangladesh and Myanmar, respectively. Spontaneous mutations that occur in local rice germplasm serve as an important source of genetic variation of glutelin seed storage units, and might be used to understand the relationship between glutelin polypeptides and the genes that encode them.