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Characterization of a new rice glutelin gene *GluD-1* expressed in the starchy endosperm

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Abstract

A new glutelin gene, designated *GluD-1*, has been discovered by comparing the seed storage proteins from 48 *japonica* and *indica* rice cultivars on SDS-PAGE gels. Evidence that GluD-1 is a member of the glutelin family was provided by Western blots using anti-glutelin antiserum and by mapping the gene to the chromosomal glutelin gene cluster. The limited GluD-1 size polymorphism among the rice varieties is due to amino acid substitutions rather than to post-transcriptional modification. *GluD-1* is maximally expressed in the starchy endosperm starting at 5 d after flowering (DAF) and increasing through 30 DAF, a major difference from the other glutelins which are primarily expressed in the subaleurone from 10–16 DAF. Only about 0.2 kb of the *GluD-1* promoter was sufficient to confer inner starchy endosperm-specific expression. The 0.2 kb truncated *GluD-1* promoter contains a bifactorial endosperm box consisting of a truncated GCN4 motif (TGA(G/C)TCA) and AAAG Prolamin box (P box), and ACGT and AACA motifs as *cis*-regulatory elements. Gel retardation assays and *trans*-activation experiments indicated that the truncated GCN4 and P box are specifically recognized by RISBZ1 b-ZIP and RPBF Dof activators *in vitro,* respectively, and are synergistically transactivated, indicating that combinatorial interactions of these motifs are involved in essential endosperm-specific regulation. Furthermore, deviation from the cognate GCN4 motif alters tissue-specific expression in the inner starchy endosperm to include other endosperm tissues.

Introduction

Rice is consumed as a staple food by about 60% of the world's population, and by more than 80% in Asian countries. Rice grain provides a major source of carbohydrates and proteins, especially in developing countries. Starch and storage proteins are predominantly deposited in the rice grain endosperm during seed maturation, and are used as a source of nitrogen and carbon for the germinating seedling.

Seed storage proteins (SSPs) can be grouped according to their physical properties based on solubility (Osborn, 1924). Albumins, globulins, prolamins, and glutelins make up the bulk of SSPs. In rice, glutelin is the major SSP, accounting for 60–80% by weight of total seed protein. Prolamin makes up about 20–30%, which is distinct from other cereal seeds, which have far more prolamin as a percentage of total protein. Glutelin and globulin are deposited in protein body II (PB-II) storage vacuoles, whereas prolamins accumulate in endoplasmic reticulum- (ER) derived protein body I (PB-I) structures that form within the lumen of the rough ER (Takaiwa *et al.*, 1999). Rice glutelin originates from the same ancestral gene as 11S globulin. Rice glutelin is synthesized as a M_r 57 000 (57 kDa) precursor protein and is transported into the PB-II via ER and Golgi or directly from ER, where it is processed into mature M_r 37 000 (37 kDa) acidic and M_r 20 000 (20 kDa) basic subunits

(Yamagata *et al.*, 1982; Furuta *et al.*, 1986; Krishnan and Okita, 1986).

Rice endosperm is an excellent platform for producing foreign recombinant proteins because rice crops are grown across very diverse agricultural regions, proteins can be produced in relatively abundant amounts, and targeted with considerable accuracy to specific tissues within the endosperm, and the proteins are stable at room temperature for long periods. A number of biopharmaceuticals, nutriceuticals containing health-promoting peptides, and mineral-binding peptides have been produced using rice endosperm as a bioreactor (Ye *et al.*, 2000; Paine *et al.*, 2005; Takagi *et al.*, 2005*a*, *b*; Takaiwa, 2007; Takaiwa *et al.*, 2007). The nutritional improvement of SSPs either by traditional breeding or by genetic engineering would be a substantial benefit to consumers because they are a major source of amino acids in the human diet, but are deficient in the essential amino acids lysine, threonine, and tryptophan. A parallel goal has been to reduce the production of native SSPs by mutation or gene suppression to allow for the production and accumulation of recombinant proteins, because low SSP mutants provide higher potential capacity for foreign gene products (Tada *et al.*, 2003). Moreover, transgenes can be highly expressed in a specific tissue during a specific developmental stage by employing native SSP promoters (Qu and Takaiwa, 2004).

Many of the molecular mechanisms underlying endosperm-specific expression of cereal SSP genes have been determined. Several *cis*-elements, such as the prolamin box (P box: AAAG) and GCN4 (TGA(G/C)TCA), ACGT, and AACA motifs are conserved in many cereal SSP genes (Zheng *et al.*, 1993; Takaiwa *et al.*, 1996; Washida *et al.*, 1999; Wu *et al.*, 2000). Transcriptional factors that recognize these *cis* elements have also been isolated, such as Opaque2-like bZIP, Dof, and Myb proteins (Schmidt *et al.*, 1992; Albani *et al.*, 1997; Vicente-Carbajosa *et al.*, 1997; Mena *et al.*, 1998; Onate *et al.*, 1999; Onodera *et al.*, 2001; Diaz *et al.*, 2002; Yamamoto *et al.*, 2006). Understanding these gene regulation mechanisms in greater detail would provide useful information for fine-tuning the expression of recombinant products in transgenic crops.

In this study, a new glutelin was discovered by comparing the seed protein composition of various rice cultivars. The expression of this glutelin gene (*GluD-1*) has unique spatial and temporal features during seed development, although, like the other glutelins, its expression is endosperm-specific and GluD-1 is deposited in PB-II storage structures. *GluD-1* was predominantly expressed in the inner starchy endosperm beginning about 5 DAF and steadily increased until maturity at 30 DAF. Such endosperm-specificity is controlled by combinatorial interactions of a GCN4-like motif and closely linked P box around position –200 from the ATG start site through binding to RISBZ1 and RPBF. Its spatial specificity may

be caused by deviations within the GCN4 motif of the *GluD-1* promoter.

Materials and methods

Plant materials

Forty-eight rice (*Oryza sativa* L.) accessions were obtained from the Genebank of the National Institute of Agrobiological Sciences (NIAS) (Table 1). Rice cultivars Nipponbare, Koshihikari, Nihonmasari, Kita-ake, Yamadanishiki, a-123, and *Lgc-1*, Koshihikari/ Nona Bokra chromosomal segment substituted lines (CSSLs), and Nipponbare/Kasalath back-cross inbred lines (BILs) obtained from the Rice Genome Resource Center (RGRP) of NIAS (Lin *et al.*, 1998; Takai *et al.*, 2007), were also used in this study. Kita-ake and Nanjing 11 were crossed and progeny were cultivated in a greenhouse.

Seed protein extraction and Western blotting

Maturing or mature seeds were ground to a fine powder with a Multi-Beads Shocker (Yasui kikai, Osaka, Japan). Seed proteins were extracted with protein extraction buffer (50 mM TRIS-HCl (pH 6.8), 4% SDS, 8 M urea, 5% 2-mercaptoethanol, and 20% glycerol) by shaking vigorously for 2 h at room temperature. Extracts were then centrifuged at 15 000 rpm for 5 min to remove debris. The anti-glutelin antibody was kindly provided by Dr S Utsumi (Kyoto University). Anti-GluD peptide antibody was produced against the synthetic peptide RQEEHRQYQQVQYR-EGQY, which is in the C-terminal region of the acidic subunit between positions 267 and 284. Antibodies against GluA, GluB, and GluC were prepared as described previously (Takagi *et al.*, 2006). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). The blots were blocked in TRIS-buffered saline plus Tween 20 TBST; 25 mM TRIS-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 containing 5% skimmed milk, and then reacted with antibodies in blocking buffer. The blots were washed three times in TBST and incubated with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody in blocking buffer. Signals were detected with an ECL kit (GE Healthcare).

Expression of recombinant GluD-1 acidic subunits in E. coli

The acidic subunit regions of *GluD-1* were amplified from Nipponbare and Wataribune 2 cDNA by PCR with forward and reverse primers containing overhanging *Nco*I and *Bam*I recognition sites, respectively. After *Nco*I and *Bam*I digestion, PCR fragments were cloned into pET15b (Novagen). The expression of recombinant GluD-1 acidic subunits was induced using OvernightExpress (Novagen) according to the manufacturer's protocol.

RNA extraction and expression analysis

Total RNA from roots, shoot apices, and leaf blades was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA from seeds at 5, 10, 15, 20, and 30 d after flowering (DAF) were extracted as described previously (Takaiwa *et al.*, 1987). After RNase-free DNase (Takara) treatment, 2.0 μg of total RNA was reverse-transcribed using the oligo(dT) primer and SuperScript III (Invitrogen). The primer sets used were 5′-TCC-ATCTTGGCATCTCTCAG-3′ and 5′-GTACCCGCATCAGGCAT-CTG-3′ for *ACTIN*, and 5′-GGATTGACTTTTCCTGGTTGCC-3′ and 5′-TTACTCTTGCAGCACCCATTCC-3′ for *GluD-1*.

To produce *PGluD-1:GUS* constructs, the 5′ flanking regions of *GluD-1* (–1679/–29, –1225/–29, –589/–29, –429/–29, and –231/–29)

Table 1. *Rice accessions from Genebank of NIAS*

Lanes in Fig. 1 (Lane no.) and stock numbers in Genebank of NIAS (Stock no.) are indicated.

Lane no.	Stock no.	Accession name	Lane no.	Stock no.	Accession name
1	6155	Yoneshiro	25	13530	IR24
2	6612	Shomokita	26	12343	Asominori
3	6868	Hatsukogane	27	10484	Wataribune 2
4	8479	Kochihibiki	28	10485	Wataribune 3
5	12536	Suweon 258	29	13503	Kaohsiung 139
6	13070	Guizhao 2	30	15910	Saturn
7	15539	Dunghan Shali	31	15884	Dawn
8	37853	Tannemochi	32	13953	Nga Cheik
9	60201	India Dular	33	15963	Toro
10	77001	Fukei 158	34	12031	Shinriki
11	88629	Kantou Mochi 157	35	10919	Funakiomachi
12	88632	Kantou Mochi 160	36	7507	Shigawatarifune 6
13	88636	Kantou Mochi 164	37	8223	Kiryouyoshi
14	95756	Fukei 161	38	42267	Habiganj Boro 2
15	95758	Fukei 163	39	16089	Dourado Agulha
16	81339	Babutong	40	6956	Nan-Ei
17	119182	Taichung Native 1	41	13592	Azucena
18	13043	Guangluai 4	42	13580	BPI 76
19	13076	Erjiuqing	43	13524	Raminad Strain 3
20	12930	Nanjing 11	44	94590	Siam 29
21	16082	Dourado	45	15594	Bomba
22	13622	IAC25	46	15595	Bomba
23	16085	Pratao Precoce	47	15920	H501
24	129373	Niaw Sampa Tong	48	15337	H501

were amplified by PCR using forward and reverse primers containing overhanging *Sal*I and *Sma*I recognition sites, respectively. PCR fragments were digested with *Sal*I and *Sma*I, and cloned into pGPTV-HPT (Becker *et al.*, 1992). The *PGluD-1:GUS* construct was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Rice transformation was performed as described by Goto *et al.* (1999). For histochemical analysis, seeds at 7, 10, and 15 DAF were sectioned with a razor blade and incubated in 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) and 20% methanol at 37 °C. Quantitative analysis of promoter strength was performed as described previously (Jefferson, 1987). Maturing seeds at 17 DAF were homogenized in GUS extraction buffer (50 mM NaPO4 [pH 7.0], 10 mM 2-mercaptoethanol, 10 mM Na_2-EDTA, 0.1% SDS, 0.1% Triton X-100), and centrifuged. Ten μl of the supernatant was mixed with 90 μl GUS assay buffer (GUS extraction buffer with 1 mM 4-methyl-umbelliferryl-β-D-glucuronide (4MUG)) and incubated at 37 °C for 60 min. Reactions were stopped with 900 μl 0.2 M Na_2CO_3. A dilution series of 4-methylumbelliferone (4MU) was used as a standard. Protein concentrations were determined by the Bradford method with a Protein assay kit (Bio-Rad), using a bovine serum albumin (BSA) dilution series as the standard. Three or four seeds from each independent transgenic plant were assayed.

Phylogenetic analysis

A BLAST search for glutelin genes was carried out against the Nipponbare genomic sequence at RAP-DB (http://rapdb.dna.affrc.go.jp/). Multiple sequence alignments were performed using ClustalW (http://clustalw.ddbj.nig.ac.jp). A phylogenetic tree was constructed using a Neighbor–Joining method (TreeView programs) (Page, 1996). The number of times that each branch topology was found during bootstrap analyses was determined and are indicated at branch points (*n*=1000) (see Supplementary Fig. S1 at *JXB* online).

Transient expression assay

The PCR product of the 5′ flanking region of *GluD-1* (–1679/–29) and (–239/–29) were cloned into pBI201 to construct *PGluD-1:GUS*. The GLM mutagenized forms, GCN4 and mGCN4, were amplified by overlapping PCR, and cloned into pBI201. *PGluA-2* (–682/+11):*GUS* and *PGluB-1*(–2290/+44):*GUS* were constructed as described previously (Yamamoto *et al.*, 2006). CaMV *P35S: RISBZ1* and *P35S:RPBF* were constructed as effecters as described by Yamamoto *et al.* (2006). Transient expression in rice callus protoplasts and enzyme assays were performed by electroporation as previously described (Wu *et al.*, 1998*b*).

Prediction of cis-elements

Putative *cis*-elements within the *GluD-1* promoter were analysed using the Plant *cis*-acting regulatory DNA elements (PLACE) database (Higo *et al.*, 1999).

Gel shift assay

The coding sequences of *RISBZ1* and *RPBF* were amplified using forward and reverse primers containing overhanging *Bam*I recognition sites. PCR products were inserted into pET15b (Novagen). His-tagged recombinant proteins were expressed in Rossetta-gami *Escherichia coli* cells (Novagen), then purified on a Ni-NTA spin column (Qiagen). *GluD-1* (–239/–118) and *GluB-1* (–199/+23) promoter fragments were amplified by PCR. P box-mutagenized forms were amplified by overlapping PCR. To generate double-stranded oligonucleotides, complementary single-stranded oligonucleotides were annealed. Primers were 5′-TATGGTATGAAT-CATTTCAAC-3′ for GLM-wt, 5′-TATGGTATGAgTCATTTCA-AC-3′ for GLM-GCN4, and 5′-TATGGTATctagaATTTCAAC-3′ for GLM-mGCN4. Lower case letters indicate mutations. Probes were labelled with DIG dUTP using terminal transferase (Roche). DNA binding reactions were performed as described previously (Onodera *et al.*, 2001).

Immunocytochemical observation

Immature 15 DAF seeds were fixed in 4% paraformaldehyde, 0.2% glutaraldehyde, and 50 mM sodium phosphate buffer (pH 7.2) overnight at 4 °C. After washing in sodium phosphate buffer, the samples were dehydrated in a graded ethanol series and embedded in LR White resin (London Resin). Ultrathin sections, prepared using an ultramicrotome and mounted on nickel grids, were blocked in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$) containing 1% BSA, then incubated with anti-GluD-1 rabbit antibody. Grids were washed in washing buffer (PBS containing 0.1% BSA, 0.5 M NaCl, and 0.05% Tween 20) and incubated with gold-labelled goat anti-rabbit IgG antibody in PBS containing 1% BSA. Grids were rewashed and fixed with PBS containing 1% glutaraldehyde, rinsed twice with distilled water, and stained with uranyl acetate and lead citrate. Sections were observed with a transmission electron microscope (H-7100, Hitachi) at an accelerating voltage of 75 kV.

Results

Seed storage proteins

Mature seed proteins extracted from 48 *japonica* and *indica* accessions were separated by SDS-PAGE (Fig. 1). Irrespective of variations in relative protein amounts contained in a grain, the composition of total seed proteins as manifest by their SDS-PAGE banding patterns were nearly identical, except for two bands with molecular weights (MW) of approximately 60 kDa and 28 kDa. The *c.* 60 kDa band is thought to be the granule-bound starch synthase (GBSS) known as WAXY. High levels of WAXY expression are characteristic of *indica* varieties, because WAXY is absent or is expressed at very low levels in nearly all *japonica* varieties (Sano, 1984). Interestingly, the *c.* 28 kDa band runs just below the major acidic subunits of glutelin and has some heterogeneity in size among the *japonica* cultivars. This band has been observed previously, and tentatively assigned to the glutelin family ('a4'; (Jahan *et al.*, 2005)).

Japonica cultivars Nipponbare, Koshihikari, Nihonmasari, Yamadanishiki, and others produce a larger a4 protein (a4J) than *indica* cultivars such as Kasalath (a4I) (Fig. 2). Among *japonica* cultivars, Wataribune 2, Wataribune 3, Funakiomachi, and Shigawataribune produce an intermediate-sized a4 (a4M) (Fig. 2). Nona Bokra had the smallest a4 (a4N; Fig. 2). Thus, at least four allelic variations in size were observed for a4.

Allelic relationship between genes encoding a4

All of the F$_1$ progeny seed from a cross between Kita-ake (a4J) as female and Nanjing 11 (a4I) as male had both a4J and a4I, and their accumulation levels were similar (Fig. 2B). In the F$_2$ population, a4 expression patterns fell into four classes: (i) only a4J was expressed; (ii) a4J and a4I were expressed at similar levels; (iii) a4J and a4I were both expressed, but with a4I at a higher level; and (iv) only a4I was expressed (Fig. 2B). Since endosperm is
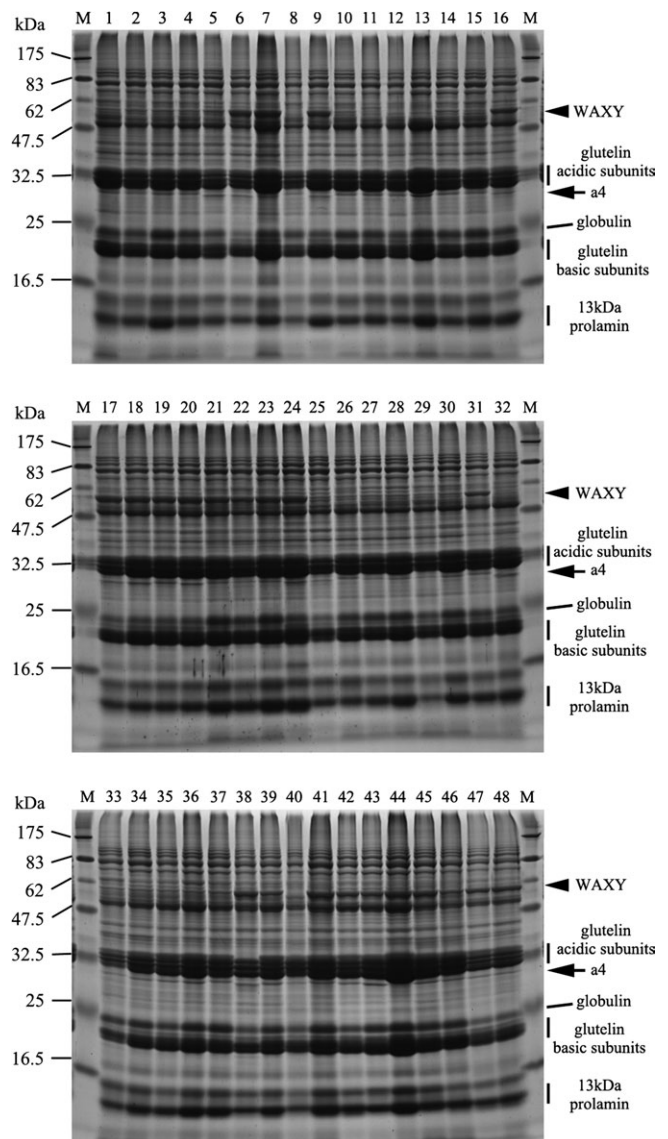

Fig. 1. SDS-PAGE analysis of seed protein composition among 48 rice accessions. Analysed accessions are listed in Table 1. Glutelin acidic/basic subunits, globulin, and 13 kDa prolamins are indicated at the right of the panels. 60 kDa (WAXY) and 28 kDa (a4:GluD-1) proteins are indicated by arrows.

triploid tissue, the difference between (ii) and (iii) results from the dosage effect. The segregation ratio of these expression classes in F$_2$ populations was 37, 31, 22, and 32, respectively, fitting to the expected ratio of 1:1:1:1 ($\chi^2=3.06$). Thus, the a4 protein is controlled by a single incomplete dominant gene.

Identification of a new glutelin

In order to determine whether the a4 band protein is a new type of glutelin, the *glu-1*, *glu-2*, and *glu-3* glutelin triple mutant (α-123), which is defective in the production of GluA-1, GluA-2, GluB-4, and GluB-5 (Iida *et al.*, 1997), and the *Low glutelin content1 (Lgc1)* mutant, which is
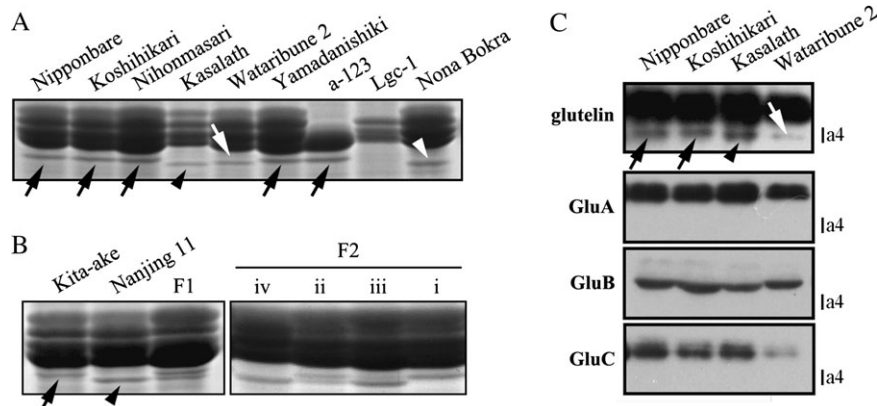
Fig. 2. SDS-PAGE analysis and Western blot analysis of allelic polymorphisms of the a4 protein band. a4J, a4I, a4M, and a4N are indicated by black arrows, black arrowheads, white arrows, and white arrowheads, respectively. (A) Allelic polymorphisms of the a4 band among cultivars Nipponbare, Koshihikari, Nihonmasari, Kasalath, Wataribune 2, Yamadanishiki, and Nona Bokra; and the a-123 and *Lgc-1* glutelin mutants. (B) Allelic interactions between a4J and a4I. F$_1$ and F$_2$ seeds of a cross between Kita-ake (a4J) and Nanjing 11 (a4I) were analysed. F$_2$ seeds segregated into one of four patterns. (C) Western blot analysis of glutelins with total seed protein from Nipponbare, Koshihikari, Kasalath, and Wataribune 2 seeds.

a dominant glutelin mutant due to RNA silencing (Kusaba *et al.*, 2003), were examined for the presence of a4. α-123 produced a4J protein, indicating that it is not GluA-1, GluA-2, GluB-4 or GluB-5. There was no a4 band in *Lgc1*, but a4J was produced by cultivar Nihonmasari, which is the wild-type background of *Lgc1* (Fig. 2A), suggesting that a4 is a glutelin. In Western blots with anti-glutelin, anti-GluA, anti-GluB, and anti-GluC antisera, anti-glutelin antibody reacted with a4J in Nipponbare and Koshihikari, a4I in Kasalath, and a4M in Wataribune 2 (Fig. 2C). By contrast, specific antibodies against GluA, GluB, and GluC did not recognize the a4 band (Fig. 2C). Taken together, a4 was identified as a different and new type of glutelin.

Mapping and molecular identification of the glutelin a4 gene

Since the size of a4 is consistently different between *japonica* and *indica* varieties, advantage was taken of chromosomal segment substituted lines (CSSLs) and back-cross inbred lines (BILs) constructed between *japonica* and *indica* cultivars. Among the 44 Koshihikari/Nona Bokra (Kos/Nona) CSSLs and their parents, Nona Bokra, SL504, SL505, and SL506 had a4N (data not shown), and only SL504, SL505, and SL506 carried a Nona Bokra segment defined by markers RM5897 and RM1313 on Chromosome 2. From a comparison of graphical genotypes and a4 phenotypes of 98 Nipponbare/Kasalath/Nipponbare (Nip/Kas/Nip) BILs (data not shown), the a4 gene mapped within a segment bounded by markers G227 and R712 (see Supplementary Fig. S2A at *JXB* online; http://www.gramene.org). Further genotyping indicated that the a4 gene was located between markers RM145 and G227, which spanned approximately 1.3 Mb (see Supplementary Fig. S2A at *JXB* online).

A BLAST search of the complete Nipponbare genome sequence indicated that there are 15 glutelin genes in the rice genome (Table 2) that can be further classified into four subfamilies, GluA, GluB, GluC, and GluD, by phylogenetic analysis (see Supplementary Fig. S2B at *JXB* online). *GluB-1a*, *GluB-1b*, *GluB-2*, *GluB-6*, and *GluD-1* are arranged in a cluster in the 1.3 Mb region bounded by markers RM145 and G227. Since GluB-1 and GluB-2 were ruled out as possibilities for a4 as described above, the genomic sequences of *GluB-6* and *GluD-1* were compared between *japonica* cultivars Nipponbare (a4J) and Wataribune2 (a4M). There were no polymorphisms in the coding region of *GluB-6* between these cultivars (data not shown). There was, however, a SNP (A173T) in the acidic subunit region of *GluD-1* which results in an amino acid substitution from Asp (Nipponbare) to Val (Wataribune 2) (see Supplementary Figs S3 and S4 at *JXB* online), and several additional SNPs between Kasalath and Nona Bokra (see Supplementary Figs S3 and S4 at *JXB* online). Anti-GluD antibody reacted with both a4J and a4M, indicating that a4 represents GluD-1 (Fig. 3).

Bacterial expression of the GluD-1 acidic subunit

To examine the possibility that differences in SDS-PAGE mobility were caused by post-translational modifications, the GluD-1 acidic subunits, including signal peptides, from cvs Nipponbare (GluD1aNip) and Wataribune 2 (GluD1aW2) were expressed in *E. coli* cells. The expressed proteins were slightly larger than the native GluD-1 acidic subunits in rice seeds due to the presence of a signal peptide (Fig. 3). Western blots were used to confirm that these proteins were GluD1aNip and GluD1aW2 (Fig. 3, lower panel). Because a difference in gel migration between GluD1aNip and GluD1aW2 was

Table 2. *Rice glutelin genes*

Located chromosomes (Chr), rice annotation project locus (RAP locus), and Accession numbers are indicated.

| Gene	Chr	RAP locus	Accession number
GluA-1	1	Os01g0762500	M17513, X05662, X05661
GluA-2	10	Os10g0400200	AK107314, X05664, X06149, X05663
GluA-3	3	Os03g0427300	AK107271, M28159
GluA-4	1	Pseudogene	–
GluB-1a	2	Os02g0249800	AK107343, X14568
GluB-1b	2	Os02g0249900	X15833
GluB-2	2	Os02g0249600	X54192
GluB-3	2	Pseudogene	X54193
GluB-4	2	Os02g0268300	X14393
GluB-5	2	Os02g0268100	AK107238
GluB-6	2	Os02g0248800	AY429651
GluB-7	2	Os02g0242600	AY196923
GluC-1	2	Os02g0453600	AK064478
GluC-2	2	Pseudogene	–
GluD-1	2	Os02g0249000	AY429650
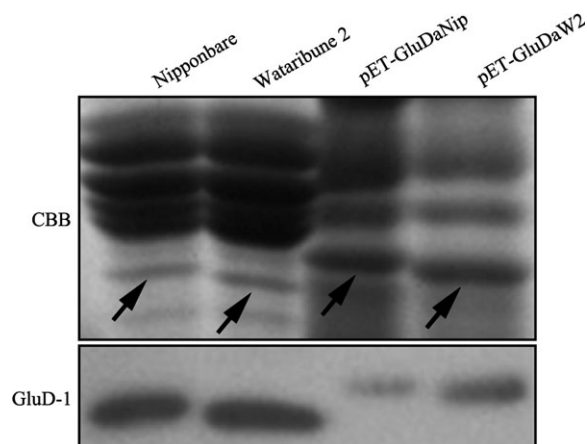
Fig. 3. Western blot analysis and bacterial expression of the GluD-1 acidic subunit. Upper panel: SDS-PAGE of native extracts from Nipponbare and Wataribune 2 seeds, and products expressed from pET-GluDaNip and pET-GluDaW2. Lower panel: Western blot analysis with anti-GluD. Arrows indicate GluD-1 acidic subunits.

Fig. 4. Temporal and spatial expression patterns of *GluD-1*. (A) Organ-specific expression pattern of *GluD-1* by RT-PCR, and GluD-1 by Western blot analysis. R, root; S, shoot apex; L, leaf blade; F, flower; Se, seed at 15 d after flowering (DAF); Ca, callus. (B) Temporal expression pattern during seed maturation of *GluD-1* by Northern blot analysis and GluD-1 by Western blot analysis. Numerals indicate sampling stage. (C–E) GUS expression driven by the 1.6 kb *GluD-1* promoter during seed maturation, at 7, 10, and 15 DAF, respectively. Al, aleurone and subaleurone layers; en, starchy endosperm; em, embryo.

observed in both bacterial expression products and in seed products, the difference in gel mobility is most likely not due to post-translational modification, but rather from the single substituted amino acid.

Tissue-specific expression of GluD-1

The expression of *GluD-1* in plant organs was determined by RT-PCR from total RNA extracted from root (R), shoot apex (S), vegetative leaf (L), flower (F), and seed at 15 DAF (Se), and from callus (Ca). *GluD-1* was specifically expressed in developing seed (Fig. 4A). *GluD-1* expression was detectable by Northern blotting in seed tissue at 5 DAF with increasing intensity through 30 DAF (Fig. 4B). This spatial- and temporal-specific expression
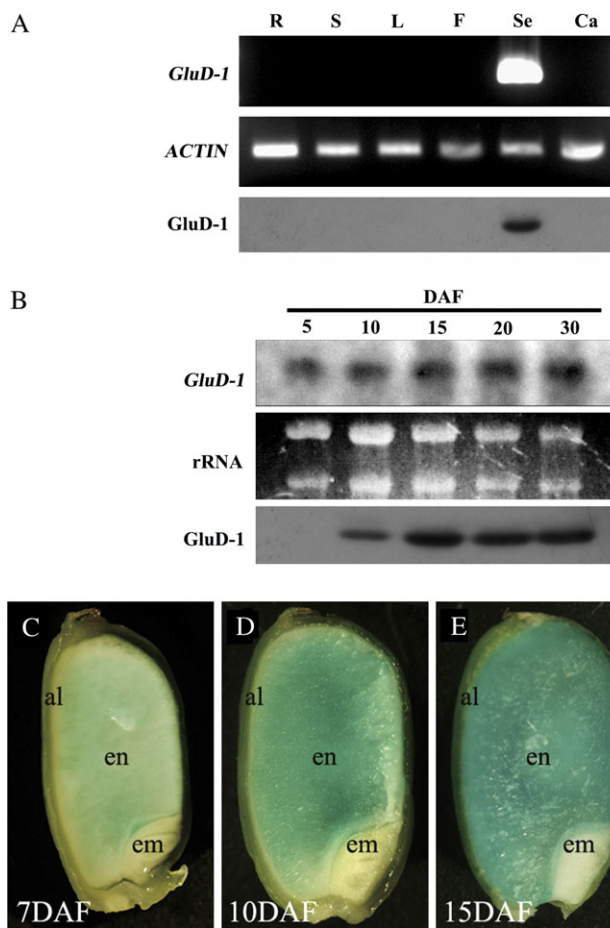
was further confirmed by Western blotting using anti-GluD-1 antibody. GluD-1 was specifically detected in mature seed, and its expression pattern was slightly different from transcription. GluD-1 was detectable by Western blotting at 10 DAF, and reached a plateau at 15 DAF (Fig. 4B).

Furthermore, to evaluate the spatial and temporal distribution of *GluD-1* within the endosperm, transgenic rice plants harbouring the GUS reporter gene under the control of the 1.6 kb *GluD-1* promoter were generated. GUS activity was observed in the inner starchy endosperm rather than in the aleurone and subaleurone layers by 7 DAF (Fig. 4C). GUS expression was less specific as the seed developed, and was found throughout the endosperm, including the aleurone and subaleurone layers by 15 DAF (Fig. 4D, E). No GUS activity was detected in

either the embryos of maturing seed or in vegetative tissues (data not shown).

Regulatory domains of the GluD-1 promoter for endosperm-specific expression

A search was made for the four SSP *cis* elements (GCN4, prolamin box (P box), ACGT, and AACA motifs) known to be responsible for endosperm expression using the Plant *cis*-acting regulatory DNA elements (PLACE) program. The 1.6 kb *GluD-1* promoter contains one GCN4 like motif (GLM), 39 P boxes, one ACGT, and four AACA motifs (Fig. 5A; Table 3). It should be noted that the GCN4-like motif (GLM) around position –200 is flanked by three P boxes (Pb1, Pb2, and Pb3), constituting an 'Endosperm box', usually containing a GCN4 motif and a P box separated by within 10 nucleotides, as has been found in many cereal prolamin promoters such as zein, hordein, and glutenin (Hammond-Kosack *et al.*, 1993; Muller and Knudsen, 1993; Marzabal *et al.*, 1998).

To characterize the *GluD-1* regulatory regions required for endosperm-specific expression further, loss-of-function experiments by progressive truncations from the 5′ end of the 1.6 kb *GluD-1* promoter were used to show that even when deleted to position –231, GUS activity in maturing seed was limited to the inner starchy endosperm close to the scutellum and in the peripheral aleurone and subaleurone layers of the abaxial side at an early maturating stage (Fig. 5B–F). Since the 0.2 kb upstream region is sufficient to confer GUS reporter expression, the essential regulatory elements controlling *GluD-1* expression in the starchy endosperm are present within this region. The average GUS activities with upstream regions of 1.6 kb, 1.2 kb, 0.6 kb, 0.4 kb, and 0.2 kb at 17 DAP were 4.1 ± 3.3, 1.2 ± 1.4, 0.9 ± 1.0, 1.3 ± 0.9, and 0.3 ± 0.3 pmoles 4MU min^{-1} mg^{-1}, respectively (Fig. 6A, right panel), indicating that the regions –1679/–1225, and –429/–231 contain positive regulatory elements.

Trans-activation of the GluD-1 promoter by RISBZ1 and RPBF

Rice SSP genes are synergistically *trans*-activated by RISBZ1 and RPBF (Yamamoto *et al.*, 2006). To determine whether *GluD-1* expression is regulated by these factors, we performed transient expression assays using rice
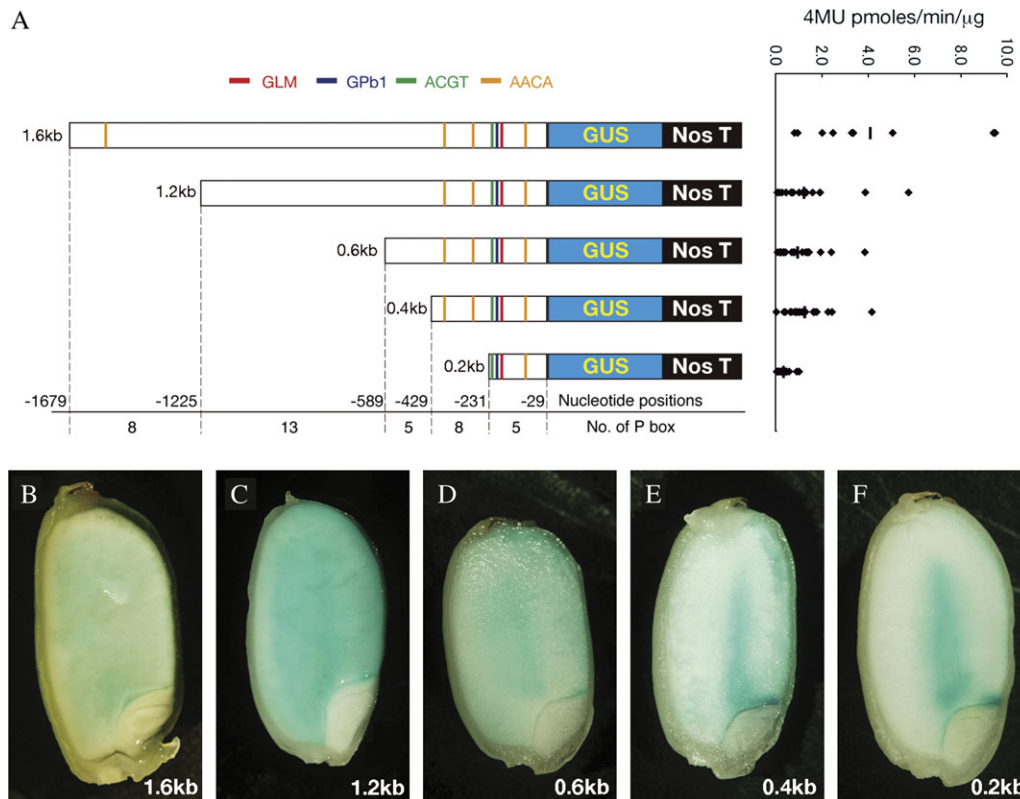
Fig. 5. Truncation analysis of the *GluD-1* promoter. (A) Schematic representation of truncated promoter:GUS constructs for generating transgenic rice (left), and expression strength (right). Promoter lengths are indicated on the left. Negative numerals indicate the nucleotide position relative to the translational start site. Putative *cis*-elements predicted by the PLACE database and this study, are indicated by colour bars. Red, blue, green, and orange bars represent GCN4-like motif (GLM), Prolamin box (P box) Pb1, ACGT, and AACA motifs, respectively. Numbers of P box within truncated regions are indicated. Promoter activities at 17 DAF are expressed as 4MU pmol min^{-1} µg^{-1} protein. Diamonds represent individual transgenic lines, and bars represent mean values. (B–F) GUS expression at 7 DAF, driven by 1.6 kb, 1.2 kb, 0.6 kb, 0.4 kb, and 0.2 kb *GluD-1* promoters, respectively. X-gluc staining reactions were carried out for 2 h (B), and for overnight (C–E), depending on GUS activity.

Table 3. *Predicted* cis-*elements in the 1.6 kb GluD-1 promoter*

cis element	Consensus	Related TF type	Positions relative to translation start site
GLM	TGA(G/C)TCA	bZIP	–194/–188[a]
P box	AAAG	DOF	–1640/–1637, –1620/–1617, –1496/–1493, –1490/–1487, –1426/–1423, –1393/–1390, –1281/–1278, –1267/–1264, –1210/–1207, –1204/–1201, –1191/–1188, –1130/–1127, –1118/–1115, –1056/–1053, –927/–924, –907/–904, –839/–836, –833/–830, –802/–799, –706/–703, –684/–681, –590/–587, –583/–580, –551/–548, –486/–4832, –477/–474, –401/–398, –369/–366, –349/–346, –343/–340, –335/–332, –328/–325, –281/–278, –252/–249, –207/–204[b], –174/–171, –157/–154, –82/–79, –75/–72
ACGT	ACGT	bZIP	–214/–211 –1500/–1495, –639/–634, –387/–382
AACA	AACAAA	Myb	–110/–105

[a] Recognized by RISBZ1.
[b] Recognized by RPBF.

callus protoplasts. Three GUS reporter constructs fused to 0.7 kb *GluA-2*, 2.3 kb *GluB-1*, and 1.6 kb *GluD-1* promoters were used to compare GUS activities upon transfection with RISBZ1 and RPBF activators. The activation of *GluA-2* (328-fold) by RISBZ1 was much greater than *GluD-1* (38-fold) and *GluB-1* (68-fold) (Table 4). By contrast, the activation of *GluB-1* (153-fold) and *GluD-1* (89-fold) by RPBF was much higher than that of *GluA-2* (13-fold) (Table 4). Co-transfection of RISBZ1 and RPBF resulted in synergistic activation of all three glutelin promoters. However, the synergistic effect of both effectors on the *GluD-1* promoter (867-fold) was much lower than on the *GluA-2* (1006-fold) and *GluB-1* promoters (2549-fold) (Table 4).

Interactions between the GluD-1 promoter and RISBZ1 and RPBF

Gel-retardation assays were used to determine whether RISBZ1 and RPBF bind directly to the GLM and the P boxes in the *GluD-1* promoter (–231/–118) (Fig. 6A). Retarded protein bands were observed after incubation with RISBZ1 or RPBF (Fig. 6B). A 100-fold molar excess of non-labelled competitor abolished these retarded bands, indicating that the *GluD-1* promoter is specifically recognized by RISBZ1 and RPBF. The retardation effect associated with RISBZ1 was apparently weaker than with RPBF, and equal amounts of protein incubated with a DIG-labelled *GluB-1* promoter (–199/+23) fragment gave much stronger retardation by RISBZ1 than with *GluD-1*. To test the specific recognition of the GLM by RISBZ1, interactions between RISBZ1 and 21 bp oligonucleotides containing the GLM within the *GluD-1* promoter (wt), the typical GCN4 (gain of function; GCN4) or the altered GCN4 (loss of function; mGCN4), were analysed (Fig. 6C). Incubation of RISBZ1 and the 21 bp GLM-GCN4 containing the cognate GCN4 motif (TGAGTCA), which is present in *GluB1* promoter and is known to be recognized by RISBZ1, strengthened the retardation band.

By contrast, use of the 21 bp GLM-mGCN4 containing mutated GCN4 abolished retardation, indicating that RISBZ1 specifically recognized the GLM of the *GluD-1* promoter, but with low affinity. This phenomenon was confirmed *in vivo* by transient expression assays using rice callus protoplasts. The activation of 0.2 kb *PGluD-1:GUS* with intact GLM by RISBZ1 (69-fold) was enhanced by gain of function (GCN4: 153-fold), and significantly decreased by loss of function (mGCN4: 16-fold) (Fig. 6D). To determine which P box was recognized by RPBF, interactions between RPBF and three fragments with altered P boxes were tested (Fig. 6B). Retardation bands were detected when RPBF was incubated with mt2 and mt3, but not with mt1, indicating that RPBF specifically recognizes P box Pb1.

Subcellular localization of GluD-1

Rice SSPs are contained in ER-derived protein body I (PB-I) structures or in storage vacuoles (PB-II). The two types of PBs can be distinguished by their size, electron density, and shape. Glutelins are predominantly localized in high density, irregularly-shaped PB-II, and prolamins are found in the low density, round PB-I bodies. Gold particle-labelled anti-GluD antibodies were mainly distributed in PB-II (Fig. 7), indicating that GluD-1 is deposited in PB-II like other glutelins.

Discussion

Rice seed endosperm is a good platform, not only for the production of recombinant proteins but also for nutriceutical delivery (Takaiwa, 2007; Takaiwa *et al.*, 2007). Most SSP genes are specifically and highly expressed in seed, and their promoters have been used to express a number of transgenes in seed (Wu *et al.*, 1998a; Qu and Takaiwa, 2004). Glutelin, accounting for 60–80% of rice seed protein, is synthesized as a 57 kDa precursor on rough
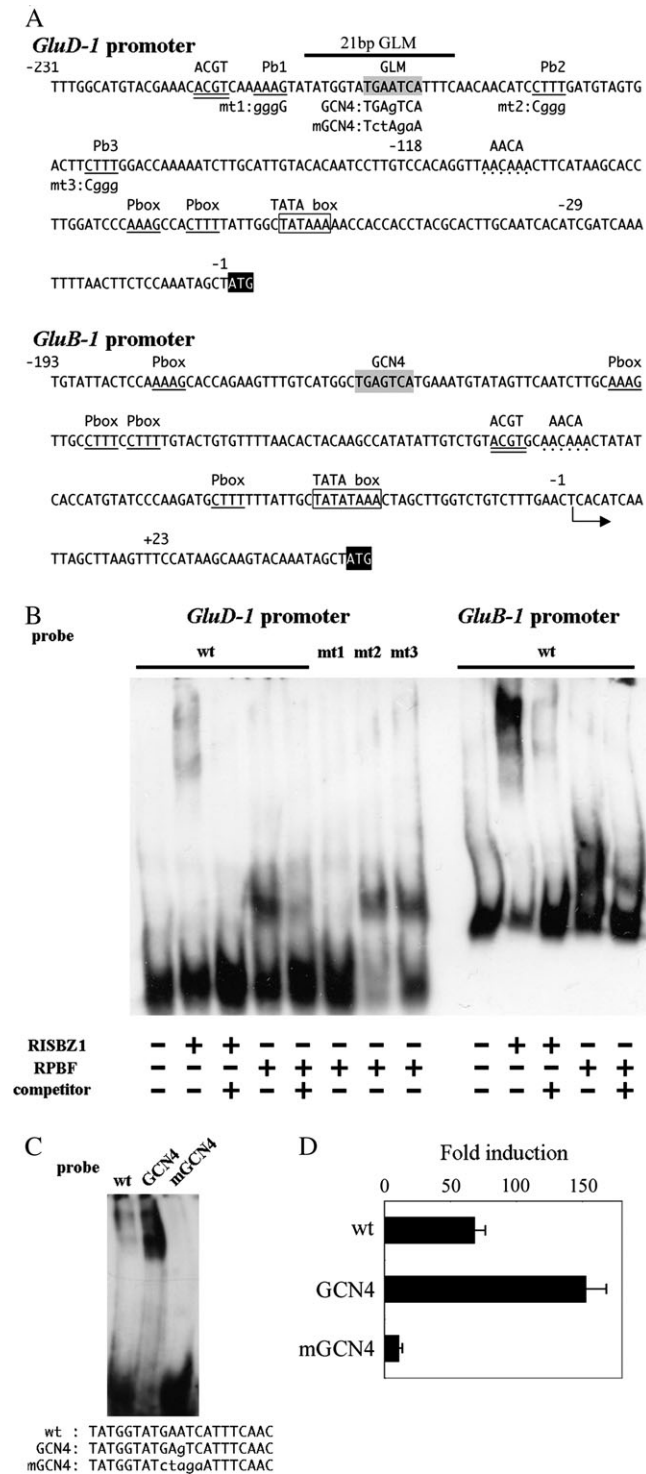
A

GluD-1 promoter

GluB-1 promoter

B

C D


```
wt    : TATGGTATGAATCATTTCAAC
GCN4  : TATGGTATGAGTCATTTCAAC
mGCN4 : TATGGTATctagaATTTCAAC
```

Fig. 6. Electro-Mobility Shift Assays (EMSA) of recombinant RISBZ1 and RPBF with the *GluD-1* and *GluB-1* promoters, and transient expression analysis of 0.2 kb *PGluD-1:GUS* and GLM-mutated *PGluD-1:GUS* constructs. (A) Sequence of the 0.2 kb *GluD-1* promoter and 0.2 kb *GluB-1* promoter. Numerals indicate the nucleotide position from the translational start site for the *GluD-1* promoter, and from the transcriptional start site for the *GluB-1* promoter. The GCN4-like motif (GLM) is shaded in grey. Prolamin boxes (P box) are underlined. Three P boxes flanking the GLM, (Pb1, Pb2, and Pb3) are numbered. Mutant forms for loss-of-function and gain-of-function analyses are indicated

by lower-case letters just below the mutated positions. The ACGT motif is double underlined. The AACA motifs are indicated by dots. A putative TATA box is framed, and the ATG start codon is shaded in black. The 21 bp oligonucleotides probe region (21 bp GLM) for EMSA is indicated. (B) EMSA of RISBZ1 and RPBF with the *GluD-1* promoter, P box-mutated *GluD-1* promoters, and *GluB-1* promoter. Competition experiments are performed using 100-fold excess molar unlabelled wild-type probes. DIG-labelled probes are indicated above each lane. (C) EMSA of RISBZ1 with 21 bp GLM oligonucleotides and mutagenized forms within *GluD-1* promoter. The oligonucleotide probes used were indicated below the panel. (D) Transient expression analysis of 0.2 kb *PGluD-1:GUS* and GLM-mutated *PGluD-1:GUS* constructs. CaMV *P35S:RISBZ1* as an effecter and 0.2 kb *PGluD-1:GUS* (wt), its gain of function version (GCN4) and loss of function version (mGCN4) as reporters are co-transfected into callus-derived protoplasts. Trans-activation levels were represented as fold induction, relative to expression of a GUS reporter gene from each promoter without any effecter.

ER, then transported into PB II storage protein vacuoles either via the Golgi apparatus, or directly, where it is processed into 37 kDa acidic and 20 kDa basic subunits (Takaiwa *et al.*, 1999). An extensive analysis of the complete rice genome sequence indicates that there are at least 11 glutelin genes (Katsube-Tanaka *et al.*, 2004). However, only GluA-1, GluB-4, and GluB-5 have been conclusively associated with their corresponding genes (Kusaba *et al.*, 2003; Qu *et al.*, 2003; Morita *et al.*, 2007).

In this study, a glutelin with a 28 kDa acidic subunit was found to accumulate in seed. This protein, named here as GluD-1, has the smallest of the glutelin acidic subunits, and GluD-1 exhibits a few size polymorphisms among the *indica* and *japonica* varieties tested (Fig. 2). Furthermore, 28 SNPs were detected in the *GluD-1* DNA sequences of four *japonica* and *indica* cultivars. The *GluD-1* SNP frequency between Nipponbare and Kasalath was about 1.9%, more than double the 0.7% for the whole rice genome (Nasu *et al.*, 2002). The amino acid (aa) sequence deduced from *GluD-1* is composed of 484 aa and has *c.* 60% homology to *GluA*, *c.* 70% homology to *GluB*, and *c.* 50% homology to *GluC*. The GluD-1 aa sequence is similar to members of the GluB subfamily, but sequence similarity to GluB members was less than among GluB subfamily proteins (largely, over 80%). Thus, GluD-1 was classified into a separate group (see Supplementary Fig. S2B at *JXB* online).

As shown by Southern blot analysis, members of the rice *GluB* subfamily form a gene cluster on chromosome 2 (Okita *et al.*, 1989; Takaiwa and Oono, 1991*b*). The *GluB* cluster is arranged in the order of *GluB-6*, *GluD-1*, *GluB-2*, *GluB-1a*, and *GluB-1b*. Tandem gene arrangements have been reported for several SSP genes. Prolamin-type SSP genes, encoding such as maize zein, rice 13 kDa prolamin, and wheat gliadin, are well known to form gene clusters (Reeves and Okita, 1987; Kim and Okita, 1988; Song *et al.*, 2001).

GluD-1 was specifically expressed in maturing endosperm like other glutelin genes. However, both temporal

Table 4. *Transient expression of glutelin promoter:GUS reporter constructs*

Trans-activation levels were represented as fold induction ±SD, relative to expression of a GUS reporter gene from each promoter without any effecter.

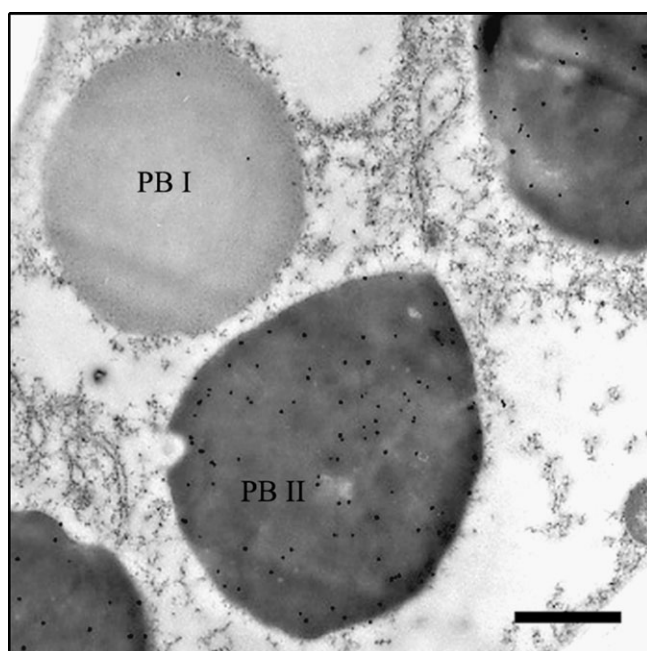| Reporter	Effector		
(Promoter:GUS)	RISBZ1	RPBF	RISBZ1+RPBF
GluA-2	32±48.8	13±0.9	1006±105.4
GluB-1	62±5.9	153±33.8	2549±419.5
GluD-1	38±3.2	89±6.4	867±60.2

Fig. 7. Intracellular localization of GluD-1 in the maturing seeds detected by immunoelectron microscopy. PB I and the high-density, irregularly shaped PB II are indicated. Twenty-five-nanometer (25 nm) gold particles labelled with anti-GluD-1 antibody are predominantly distributed in PB-II. Bars=1μ m.

and spatial expression patterns of *GluD-1* within the endosperm were different from other glutelin genes. During seed development, mRNA levels of *GluD-1* gradually increased at least until the mature stage 30 DAF, which is in contrast to the mRNA levels of many other glutelin genes, which start to increase at 5 DAF, reach a peak between 10 and 16 DAF, and then gradually decrease during seed development (Okita *et al.*, 1989; Takaiwa and Oono, 1990; Takaiwa and Oono, 1991*a*; Takaiwa *et al.*, 1991). Differential temporal expression patterns have also been observed among the soybean glycinin and maize zein families (Domoney and Casey, 1987; Kirihara *et al.*, 1988). Furthermore, the 1.6 kb *GluD-1* promoter directed GUS expression in the inner

starchy endosperm at an early maturing stage, which would be complementary to other glutelin gene promoters (Qu and Takaiwa, 2004).

Because of the current initiatives to exploit plants as bioreactors for the production of various pharmaceutical and industrial recombinant proteins (Takaiwa, 2007), it has become apparent that several promoters could be used to drive expression in the starchy endosperm. The *Glb-1* promoter, in particular, gives quite strong expression in the starchy endosperm (i.e. 43.5 [4MU pmol min^{-1} μg^{-1} protein]) (Wu *et al.*, 1998*a*). However, expression of recombinant products by the *Glb-1* promoter sometimes causes detrimental effects on growth, because *Glb-1* is also expressed in vegetative tissues (Wu *et al.*, 1998*a*). *GluD-1*, however, is specifically expressed in the endosperm, making it more suitable for expressing recombinant proteins in the starchy endosperm. The *RAG-1* promoter also drives expression only in the starchy endosperm, but its expression is lower than that of the *GluD-1* promoter (approximately 0.9 versus 4.1 [4MU pmol min^{-1} mg^{-1} protein]) (Wu *et al.*, 1998*a*).

Several *cis*-elements, such as GCN4, prolamin box (P box), ACGT, and AACA motifs are conserved among various cereal SSP gene promoters and are involved in endosperm-specific expression (Zheng *et al.*, 1993; Washida *et al.*, 1999; Wu *et al.*, 2000). In rice, the GCN4 motif in the proximal region of the *GluB-1* promoter is recognized by RISBZ1 activator and acts as a critical *cis*-element for the expression in the aleurone and subaleurone layers, because multimers of the GCN4 motif can direct endosperm-specific expression (in the aleurone and subaleurone layers) in stable transgenic rice (Wu *et al.*, 1998*b*; Wu *et al.*, 2000; Onodera *et al.*, 2001). On the other hand, multimers of the P box, ACGT, and AACA motifs cannot direct endosperm-specific expression, indicating that these elements may be involved in quantitative regulation by combinatorial interaction with the GCN4 motif in the endosperm (Wu *et al.*, 2000). Furthermore, since mutations in individual motifs in a minimal *GluB-1* promoter for the endosperm-specific expression abolished or severely suppressed GUS expression in endosperm, it has been suggested that combinatorial interactions of these four motifs are required for expression to take place in the endosperm (Wu *et al.*, 1998*b*, 2000). Like these other promoters such as *GluA-2* and *GluB-1* promoter, the *GluD-1* promoter directed endosperm-specific expression, but predominantly in the inner starchy endosperm, even though four *cis*-elements, essential for endosperm-specific glutelin gene expression, are present in the *GluD-1* promoter. 0.2 kb of the *GluD-1* promoter, which contains all four *cis*-elements, was sufficient to confer endosperm-specific expression (Fig. 6A).

Gel retardation assays and *trans*-activation experiments clearly demonstrate that the GCN4-like motif and P box in

the *GluD-1* promoter region –231/–29 were specifically recognized by RISBZ1 and RPBF (Fig. 6B, C; Table 3). It is important to note that activation of the *GluD-1* promoter by RISBZ1 was lower than activation of the *GluA-2* and *GluB-1* promoters, a difference which can be accounted for by low affinity binding due to deviations in the GCN4 motif sequence from the cognate GCN4 (i.e. TGA(G/C)TCA to TGAATCA), resulting in poor RISBZ1 recognition of the *GluD-1* promoter GLM (Fig. 6). The expression site also changed to the inner starchy endosperm when the mutation was introduced into the GCN4 motif in the 197 bp *GluB-1* promoter to include aleurone and sub-aleurone layer-specific expression (Wu *et al.*, 1998). It has recently been shown that co-suppression of *RPBF* in maturing seed also decreased *GluD-1* expression, whereas co-suppression of *RISBZ1* had little effect on *GluD-1* expression, indicating that RPBF contributes significantly to *GluD-1* expression *in planta* (MP Yamamoto *et al.*, unpublished results). Notably, knockout/knockdown mutants of transcription factors often exhibit only subtle phenotypes, due to functional redundancy of transcription factors that belong to the same family (Hiratsu *et al.*, 2003; Zhang, 2003). It has been reported that *RISBZ2-5*, related to *RISBZ1*, are also expressed in the endosperm (Onodera *et al.*, 2001). Although these other bZIP proteins *trans*-activate the expression from GCN4 much less than RISBZ1, it is possible that they participate in the regulation of *GluD-1* expression and compensate for RISBZ1 absence. Furthermore, rice allergenic *RAG-1* and *Glb-1* promoters are expressed in the inner starchy endosperm. There is no cognate GCN4 motif in these promoters, although truncated GCN4 motifs in the *Glb-1* promoter are recognized by RISBZ1 *in vitro*. Furthermore, these promoters are not activated by RISBZ1, or the expression level is relatively low (Yamamoto *et al.*, 2006). Taken together, the absence or truncation of the GCN4 motif from the minimal proximal promoter, which is required for endosperm-specific expression, may be associated with the observed changes in expression pattern from the aleurone and sub-aleurone to inner starchy endosperm. Another possible explanation is that an alternative motif may act as a substitute to confer expression in the starchy endosperm by interacting with other essential *cis*-elements, since combination among P box, AACA, and ACGT motifs was not sufficient to confer endosperm-specific expression.

On the other hand, 5′ deletion analysis of the *GluD-1* promoter indicated that –1679/–1225 and –429/–231 contain enhancer domains (Fig. 5). Within these regions, one AACA (–1500/–1495) and eight P boxes, including one that is well-conserved within the endosperm box (TGCAAAG: –1270/–1264); and two AACA motifs (–639/–634 and –387/–382) are present as candidate motifs. These *cis*-elements may be responsible for quantitative regulation of *GluD-1*.

Further work will be required to determine whether the GCN4 motif acts as a critical element for determining aleurone and subaleurone-specific expression. Ideally, the relationship between the GCN4 motif and tissue-specific expression would be examined by using transgenic rice plants containing minimal endosperm-specific promoters with mutagenized GCN4 motifs.

Supplementary data

Supplementary data can be found at *JXB* online.

Fig. S1. Alignment of rice glutelin genes used for drawing a phylogenetic tree.

Fig. S2. Mapping of glutelin a4 gene and a phyllogenetic tree of rice glutelins.

Fig. S3. Alignment of *GluD-1* cDNAs among cultivars Nipponbare, Wataribune 2, Kasalath and Nona Bokra.

Fig. S4. Alignment of GluD-1 among cultivars Nipponbare, Wataribune 2, Kasalath, and Nona Bokra.

Acknowledgements

References

Albani D, Hammond-Kosack MC, Smith C, Conlan S, Colot V, Holdsworth M, Bevan MW. 1997. The wheat transcriptional activator SPA: a seed-specific bZIP protein that recognizes the GCN4-like motif in the bifactorial endosperm box of prolamin genes. *The Plant Cell* **9,** 171–184.

Becker D, Kemper E, Schell J, Masterson R. 1992. New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Molecular Biology* **20,** 1195–1197.

Diaz I, Vicente-Carbajosa J, Abraham Z, Martinez M, Isabel-La Moneda I, Carbonero P. 2002. The GAMYB protein from barley interacts with the DOF transcription factor BPBF and activates endosperm-specific genes during seed development. *The Plant Journal* **29,** 453–464.

Domoney C, Casey R. 1987. Changes in legumin messenger RNAs throughout seed development in *Pisum sativum* L. *Planta* **170,** 562–566.

Furuta M, Yamagata H, Tanaka K, Kasai Z, Fujii S. 1986. Cell-free synthesis of the rice glutelin precursor. *Plant and Cell Physiology* **27,** 1201–1204.

Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F. 1999. Iron fortification of rice seed by the soybean ferritin gene. *Nature Biotechnology* **17,** 282–286.

Hammond-Kosack MC, Holdsworth MJ, Bevan MW. 1993. *In vivo* footprinting of a low molecular weight glutenin gene (LMWG-1D1) in wheat endosperm. *EMBO Journal* **12,** 545–554.

Higo K, Ugawa Y, Iwamoto M, Korenaga T. 1999. Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research* **27,** 297–300.

Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M. 2003. Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. *The Plant Journal* **34,** 733–739.

Iida S, Kusaba M, Nishio T. 1997. Mutants lacking glutelin subunits in rice: mapping and combination of mutated glutelin genes. *Theoretical and Applied Genetics* **94,** 177–183.

Jahan M, Uemura Y, Kumamaru T, Hamid A, Satoh H. 2005. Genetic variation of glutelin acidic subunit polypeptides in Bangladesh rice genetic resources. *Genetic Resources and Crop Evolution* **52,** 977–987.

Jefferson RA. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reports* **5,** 387–405.

Katsube-Tanaka T, Duldulao JBA, Kimura Y, Iida S, Yamaguchi T, Nakano J, Utsumi S. 2004. The two subfamilies of rice glutelin differ in both primary and higher-order structures. *Biochimica et Biophysica Acta-Proteins and Proteomics* **1699,** 95–102.

Kim WT, Okita TW. 1988. Structure, expression, and heterogeneity of the rice seed prolamines. *Plant Physiology* **88,** 649–655.

Kirihara JA, Hunsperger JP, Mahoney WC, Messing JW. 1988. Differential expression of a gene for a methionine-rich storage protein in maize. *Molecular and General Genetics* **211,** 477–484.

Krishnan HB, Okita TW. 1986. Structural relationship among the rice glutelin polypeptides. *Plant Physiology* **81,** 748–753.

Kusaba M, Miyahara K, Iida S, Fukuoka H, Takano T, Sassa H, Nishimura M, Nishio T. 2003. *Low glutelin content1*: a dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice. *The Plant Cell* **15,** 1455–1467.

Lin SY, Sasaki T, Yano M. 1998. Mapping quantitative trait loci controlling seed dormancy and heading date in rice, *Oryza sativa* L., using backcross inbred lines. *Theoretical and Applied Genetics* **96,** 997–1003.

Marzabal P, Busk PK, Ludevid MD, Torrent M. 1998. The bifactorial endosperm box of gamma-zein gene: characterisation and function of the Pb3 and GZM *cis*-acting elements. *The Plant Journal* **16,** 41–52.

Mena M, Vicente-Carbajosa J, Schmidt RJ, Carbonero P. 1998. An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm. *The Plant Journal* **16,** 53–62.

Morita R, Kusaba M, Lida S, Nishio T, Nishimura M. 2007. Knockout of glutelin genes which form a tandem array with a high level of homology in rice by gamma irradiation. *Genes and Genetic Systems* **82,** 321–327.

Muller M, Knudsen S. 1993. The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. *The Plant Journal* **4,** 343–355.

Nasu S, Suzuki J, Ohta R, Hasegawa K, Yui R, Kitazawa N, Monna L, Minobe Y. 2002. Search for and analysis of single nucleotide polymorphisms (SNPs) in rice (*Oryza sativa, Oryza rufipogon*) and establishment of SNP markers. *DNA Research* **9,** 163–171.

Okita TW, Hwang YS, Hnilo J, Kim WT, Aryan AP, Larson R, Krishnan HB. 1989. Structure and expression of the rice glutelin multigene family. *Journal of Biological Chemistry* **264,** 12573–12581.

Onate L, Vicente-Carbajosa J, Lara P, Diaz I, Carbonero P. 1999. Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 *in vivo* and activates transcription from the GCN4-like motif of B-hordein promoters in barley endosperm. *Journal of Biological Chemistry* **274,** 9175–9182.

Onodera Y, Suzuki A, Wu CY, Washida H, Takaiwa F. 2001. A rice functional transcriptional activator, RISBZ1, responsible for endosperm-specific expression of storage protein genes through GCN4 motif. *Journal of Biological Chemistry* **276,** 14139–14152.

Osborn T. 1924. *The vegetable proteins*. London, FL: Longmans, Green and Co.

Page RD. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12,** 357–358.

Paine JA, Shipton CA, Chaggar S, et al. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nature Biotechnolgy* **23,** 482–487.

Qu LQ, Takaiwa F. 2004. Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. *Plant Biotechnology Journal* **2,** 113–125.

Qu LQ, Wei XL, Satoh H, Kumamaru T, Ogawa M, Takaiwa F. 2003. Biochemical and molecular characterization of a rice glutelin allele for the GluA-1 gene. *Theoretical and Applied Genetics* **107,** 20–25.

Reeves CD, Okita TW. 1987. Analyses of α/β-type gliadin genes from diploid and hexaploid wheats. *Gene* **52,** 257–266.

Sano Y. 1984. Differential regulation of waxy gene-expression in rice endosperm. *Theoretical and Applied Genetics* **68,** 467–473.

Schmidt RJ, Ketudat M, Aukerman MJ, Hoschek G. 1992. Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD-zein genes. *The Plant Cell* **4,** 689–700.

Song RT, Llaca V, Linton E, Messing J. 2001. Sequence, regulation, and evolution of the maize 22-kD alpha zein in gene family. *Genome Research* **11,** 1817–1825.

Tada Y, Utsumi S, Takaiwa F. 2003. Foreign gene products can be enhanced by introduction into low storage protein mutants. *Plant Biotechnology Journal* **1,** 411–422.

Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, Ishimitsu R, Kawauchi H, Kiyono H, Takaiwa F. 2005*a*. A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proceedings of the National Academy of Sciences, USA* **102,** 17525–17530.

Takagi H, Hirose S, Yasuda H, Takaiwa F. 2006. Biochemical safety evaluation of transgenic rice seeds expressing T cell epitopes of Japanese cedar pollen allergens. *Journal of Agricultural and Food Chemistry* **54,** 9901–9905.

Takagi H, Saito S, Yang L, Nagasaka S, Nishizawa N, Takaiwa F. 2005*b*. Oral immunotherapy against a pollen allergy using a seed-based peptide vaccine. *Plant Biotechnology Journal* **3,** 521–533.

Takai T, Nonoue Y, Yamamoto SI, Yamanouchi U, Matsubara K, Liang ZW, Lin HX, Ono N, Uga Y, Yano M. 2007. Development of chromosome segment substitution lines derived from backcross between indica donor rice cultivar 'Nona bokra' and japonica recipient cultivar 'Koshihikari'. *Breeding Science* **57,** 257–261.

Takaiwa F. 2007. Transgenic rice seed as a nutriceutical delivery system. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* **2,** 1–9.

Takaiwa F, Kikuchi S, Oono K. 1987. A rice glutelin gene family: a major type of glutelin messenger-rnas can be divided into 2 classes. *Molecular and General Genetics* **208,** 15–22.

Takaiwa F, Ogawa M, Okita TW. 1999. Rice glutelins. In: Shewry P, Casey R, eds. *Seed proteins*. Dordrecht, FL: Kluwer Academic Publishers, 401–426.

Takaiwa F, Oono K. 1990. Interaction of an immature seed-specific trans-acting factor with the 5′ upstream region of a rice glutelin gene. *Molecular and General Genetics* **224,** 289–293.

Takaiwa F, Oono K. 1991*a*. Genomic DNA-sequences of 2 new genes for new storage protein glutelin in rice. *Japanese Journal of Genetics* **66,** 161–171.

Takaiwa F, Oono K. 1991*b*. Restriction-fragment-length-polymorphism of glutelin from cultivated rice (*Oryza sativa* L). *Japanese Journal of Genetics* **66,** 155–160.

Takaiwa F, Oono K, Wing D, Kato A. 1991. Sequence of 3 members and expression of a new major subfamily of glutelin genes from rice. *Plant Molecular Biology* **17,** 875–885.

Takaiwa F, Takagi H, Hirose S, Wakasa Y. 2007. Endosperm tissue is a good production platform for artificial recombinant proteins in transgenic rice. *Plant Biotechnology Journal* **5,** 84–92.

Takaiwa F, Yamanouchi U, Yoshihara T, Washida H, Tanabe F, Kato A, Yamada K. 1996. Characterization of common *cis*-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin multigene family. *Plant Molecular Biology* **20,** 1207–1221.

Vicente-Carbajosa J, Moose SP, Parsons RL, Schmidt RJ. 1997. A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. *Proceedings of the National Academy of Sciences, USA* **94,** 7685–7690.

Washida H, Wu CY, Suzuki A, Yamanouchi U, Akihama T, Harada K, Takaiwa F. 1999. Identification of *cis*-regulatory elements required for endosperm expression of the rice storage protein glutelin gene GluB-1. *Plant Molecular Biology* **40,** 1–12.

Wu CY, Adachi T, Hatano T, Washida H, Suzuki A, Takaiwa F. 1998*a*. Promoters of rice seed storage protein genes direct endosperm-specific gene expression in transgenic rice. *Plant and Cell Physiology* **39,** 885–889.

Wu CY, Suzuki A, Washida H, Takaiwa F. 1998*b*. The GCN4 motif in a rice glutelin gene is essential for endosperm-specific gene expression and is activated by Opaque-2 in transgenic rice plants. *The Plant Journal* **14,** 673–683.

Wu CY, Washida H, Onodera Y, Harada K, Takaiwa F. 2000. Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal *cis*-element requirements for endosperm-specific gene expression. *The Plant Journal* **23,** 415–421.

Yamagata H, Tanaka K, Kasai Z. 1982. Evidence for a precursor form of rice glutelin subunits. *Agricultural and Biological Chemistry* **46,** 321–322.

Yamamoto MP, Onodera Y, Touno SM, Takaiwa F. 2006. Synergism between RPBF Dof and RISBZ1 bZIP activators in the regulation of rice seed expression genes. *Plant Physiology* **141,** 1694–1707.

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287,** 303–305.

Zhang JZ. 2003. Overexpression analysis of plant transcription factors. *Current Opinion in Plant Biology* **6,** 430–440.

Zheng Z, Kawagoe Y, Xiao S, Li Z, Okita T, Hau TL, Lin A, Murai N. 1993. 5′ distal and proximal *cis*-acting regulator elements are required for developmental control of a rice seed storage protein glutelin gene. *The Plant Journal* **4,** 357–366.