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Original Paper

Suppressive expression of the glutelin A1-collagen peptide fusion gene in hybrid rice lines with the mutant line, Low Glutelin Content-1 (LGC-1)

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Abstract A low glutelin-content mutant, LGC-1, has a mutant gene (*lgc1*) comprising a tail-to-tail inverted repeat of two highly homologous glutelin B genes (*gluB4* and *δ-gluB5*), resulting in the production of double-stranded glutelin B RNA. Hybrid rice lines between LGC-1 and a transgenic line with the glutelin A1-collagen peptide fusion gene (*gluA1-coll*) were produced, and F_2 hybrid lines homozygous for each or both of the *gluA1-coll* and *lgc1* genes were selected. The expression of *gluA1-coll* in F_2 lines with *lgc1* was far lower than that of F_2 lines without *lgc1* at both the mRNA and protein levels as measured by semi-quantitative RT-PCR and immunoblotting using anti-collagen peptide antibody. The *gluA1* mRNA sequence alignment identical to the potential double-stranded RNA formed by *gluB4* sense and *δ-gluB5* antisense RNAs consisted of 14 nucleotides at most, and was not more than 23 even when one mismatch was allowed within the sequence. Thus, such shorter double-stranded glutelin B RNAs were suggested to suppress the expression of not only endogenous *gluA* genes as reported previously (Kusaba et al. 2003) but also the exogenous *gluA1-coll* in the hybrid lines.

Key words: Glutelin multigene family, low glutelin-content mutant, RNA interference, transgenic rice, type II-collagen.

Recently, molecular farming for production of valuable proteins for pharmaceuticals, antibodies, and vaccines has been performed extensively using various agricultural crops (Ma et al. 2005). In rice, endosperm is an edible tissue and also a good production platform for artificial recombinant proteins (Takaiwa et al. 2007). Many transgenic rice plants having the ability to produce functional substances for human nutrition and health benefits are already being exploited (Paine et al. 2005; Qu et al. 2005; Takagi et al. 2005a). For successful expression and accumulation of target proteins in endosperm of transgenic rice seeds, several expression gene constructs have been developed, in which the target genes are often fused with the gene of the most abundant endosperm protein, glutelin (Wakasa et al. 2006; Yang et al. 2006). Particularly for peptide production, the expression and accumulation of glutelin-fusion protein in protein bodies would offer an advantage of stable accumulation without proteolytic degradation. In fact, some immunodominant peptides have been successfully expressed and accumulated in transgenic rice endosperm as glutelin fusion proteins, the peptides being inserted either internally into the variable region or linked to the C-terminal end of the glutelin sequence (Takagi et al. 2005b; Yasuda et al. 2006; Yang et al. 2007).

The low glutelin-content mutant, LGC-1, is known to express and accumulate glutelin in endosperm at levels much lower than wild-type rice lines (Iida et al. 1993). This lower protein-content characteristic, i.e. a higher metabolic potential for protein biosynthesis, has been considered to offer some advantages in terms of expression and accumulation of introduced genes and their translation products. By using this approach, soybean glycinin, a member of the glutelin gene superfamily, has been expressed and accumulated in the protein bodies of hybrid rice lines between LGC-1 and the glycinin-transgenic line, and the glycinin content of the LGC-1 hybrid rice seeds was reported to be 1.7 times higher than that of the original transgenic line (Tada et al. 2003).

Abbreviations: CII, Type II-collagen; *gluA1-coll*, glutelin A1-collagen peptide fusion gene; *hph*, hygromycin phosphotransferase gene (hygromycin-resistance gene); LGC-1, low glutelin content-1; RT-PCR, reverse transcriptase polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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The molecular basis of the low glutelin-content mutation has been clarified by Kusaba et al. (2003). In LGC-1, a 3.5-kb deletion between the *gluB4* and *gluB5* genes, located with an inverted orientation and sharing 99.8% nucleotide sequence identity, results in the production of a tail-to-tail inverted repeat of the two *gluB* genes. Transcription of this fused *gluB4-δgluB5* gene (*lgc1*, accession number AB093593) produces an mRNA with *gluB4* sense and *δ-gluB5* antisense sequences, which forms a hairpin structure with an intramolecular double-stranded RNA in the complementary region. This double-stranded RNA is considered to induce RNA interference against transcripts of the *gluB* gene subfamily (Takaiwa et al. 1991), resulting in remarkable suppression of GluB protein accumulation in LGC-1 (Kusaba et al. 2003). Furthermore, the expression of the *gluA* gene subfamily with 60–70% nucleotide sequence similarities to *gluB4*/*gluB5* also showed a severalfold reduction in LGC-1 compared with the parental line, Nihonmasari (Kusaba et al. 2003).

We have recently obtained genetically stable transgenic rice lines, into which a glutelin A1 gene fused with a short cDNA encoding immunodominant peptides of type II collagen (CII peptide) had been introduced (Hashizume et al. 2008). To increase the accumulation of GluA1-CII fusion protein in rice endosperm, we then attempted to breed hybrid lines of the *gluA1-coll* transgenic line with LGC-1, expecting possible advantages of its higher metabolic potential for protein biosynthesis despite a disadvantage of the severalfold reduction of *gluA* genes' transcripts in LGC-1. Another aim of this study was to determine whether the *gluB4-δgluB5*-derived double-stranded RNA affects the expression of the exogenous transgenes fused with *gluA1* gene and the accumulation of GluA1-CII fusion proteins by using the fused CII peptide tag as a reporter. Similarly to that of endogenous *gluA* genes, expression of the *gluA1-coll* fusion gene was markedly suppressed in homozygous hybrid lines with *lgc1*. The relationship between the sequence similarity of the double-stranded RNA and suppression of the transgenic *gluA1* as well as endogenous *gluA* subfamily member genes was also examined.

Materials and methods

Materials

A genetically stable transgenic rice line, into which a single copy of a glutelin-collagen fusion gene (*gluA1-coll*; glutelin A1 cDNA [accession number: NM_001050863] fused with a DNA fragment encoding immunodominant peptides of type II collagen) had been introduced, was established as described previously (Hashizume et al. 2008). A mutant rice line, the low glutelin-content 1 (LGC-1) line, which expresses and accumulates glutelin in the endosperm at levels much lower than wild-type rice lines, was a generous gift from Dr. Makoto Kusaba (The University of Tokyo). Structures of the *gluA1-coll* transgene and the mutant *lgc1* gene (Kusaba et al. 2003) are shown schematically in Figure 1.

Development of F_1 hybrid lines and selection of homozygous progeny

A *gluA1-coll* transgenic line (No. 808-55) was crossed with a low glutelin-content mutant, LGC-1, by cross-pollination using No. 808-55 as a pollen parent. The resulting F_1 hybrid lines were self-pollinated to produce F_2 progeny lines, and their zygosity was characterized by genomic-DNA PCR for both of the *gluA1-coll* and *lgc1* genes to select progeny homozygous for both genes.

Genomic DNA analyses

Genomic DNA samples were prepared from young rice leaves as described previously (Hashizume et al. 2006), and used as a template (5–50 ng DNA in 1 μl per tube) for PCR for the amplification of specific regions of transgenes. The primer pairs used for the PCR were as follows. For the 1.5-kb DNA fragment of *lgc1* (shown as PCR-3 in Figure 1B): forward, 5′-AGT-TGT-TGC-TCT-ATA-TGT-CTT-CGA-CT-3′; reverse, 5′-CTC-CTA-GAT-ATC-AAC-AAC-AGA-C-3′. For the 440-bp DNA fragment of *gluA1-coll* (shown as PCR-2 in Figure 1A): forward, 5′-CTC-AGA-GGC-TCA-AGC-ATA-ATA-GAG-G-3′; reverse, 5′-GAG-CTC-CTA-CTC-GAG-ATA-CTT-TGG-G-3′. The amplification reaction was done in a 10-μl aliquot using Ex-Taq DNA polymerase (TaKaRa BIO, Japan) in accordance with the attached manual. After being preheated at 96°C for 2 min, the DNA was denatured at 94°C for 30 s and the primers were annealed at 62°C for 1 min, and then the enzyme reaction was conducted at 72°C for 2 min. This reaction cycle was repeated 35 times in a PCR Thermal Cycler MP (TaKaRa BIO, Japan). The resulting PCR products were analyzed by electrophoresis in a 1.5% agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

For the genomic DNA blotting analysis, genomic DNA (10 μg) was isolated from leaves and digested with *Hind*III, fractionated through 0.6% agarose gels, and transferred to positively charged nylon membranes (Roche Diagnostics, Germany). The membranes were prehybridized for 30 min and then hybridized with DIG-labeled probes (the 1.5-kb *lgc1* and 440-bp *gluA1-coll* DNA fragments described above) at 42°C overnight. The membranes were successively washed in 2X SSC and 0.1X SSC at 42°C for 15 min, and this washing process was repeated again. After washing, the hybridized signal was detected using a DIG Luminescence Detection Kit (Roche Diagnostics, Germany) and exposure to Hyperfilm™ ECL (GE Healthcare Biosciences, USA).

RT-PCR analysis

Total RNA was extracted and purified from maturing rice seeds (15 DAF) using an RNeasy Plant Mini Kit (QIAGEN, USA), and mRNAs were reverse-transcribed in accordance with the manufacturer's instructions (TaKaRa One Step RNA PCR Kit (AMV), TaKaRa BIO, Japan). PCR was done using the reverse-transcribed cDNA pool as a template and primer sets specific for the 440-bp fragment of *gluA1-coll* cDNA as above (Figure 1A), a 3′-UTR-containing a 1,370-bp fragment of *gluA1* cDNA

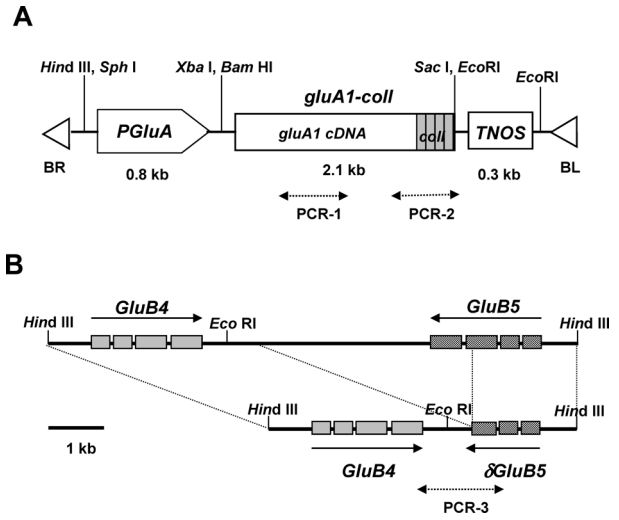

Figure 1. Schematic drawing of the *gluA1-coll* transgene (A) and the *lgc1* mutant gene (B). The *PGluA-gluA1-coll* fusion gene in the transgenic rice plant (No. 808-55) consisted of a 0.8-kb fragment (nucleotides 1568 to 2395 of accession D00584) containing the rice glutelin A promoter region (*PGluA*), *glutelin A1* cDNA in-frame fused with the DNA fragment encoding tandem repeats of CIIX4, and a 0.3-kb fragment containing the nopaline synthase terminator, *TNOS*. The detailed structure of the *gluA1-coll* connection region has been reported by Hashizume et al. (2008). Three dotted lines with arrowheads indicate the positions of DNA fragments amplified by PCR for mRNA accumulation analyses of the *gluA1* and *gluA1-coll* genes (PCR-1 and PCR-2, respectively) (Figure 4) and for preparation of DIG-labeled DNA probes used in the genomic DNA blotting analysis (PCR-2 and PCR-3) (Figure 5). BR and BL represent the right and left border sequences of the T-DNA region, respectively. Structures of the *GluB4-δGluB5* mutant gene in LGC-1 as well as the region containing the *GluB4* and *GluB5* genes in the parental rice cultivar, Nihonmasari, are shown schematically in panel B, according to the previous report (Kusaba et al. 2003).

(forward, 5′-TTC-TTC-GAT-GTC-TCT-AAT-GAG-CAA-3′; reverse, 5′-AGA-CTG-GAC-AGT-ACA-TAG-CAG-CAA-3′), another 420-bp fragment of *gluA1* cDNA located within its ORF (shown as PCR-1 in Figure 1A) (forward, 5′-TTC-TTC-GAT-GTC-TCT-AAT-GAG-CAA-3′; reverse, 5′-AGC-TAA-CAA-GAA-ATC-CCT-TTG-CCT-3′), and a 220-bp fragment of ubiquitin cDNA (forward, 5′-AAT-CAG-CCA-GTT-TGG-TGG-AGC-TG-3′; reverse, 5′-ATG-CAA-ATG-AGC-AAA-TTG-AGC-ACA-3′). After a transcriptional reaction at 50°C for 30 min and preheating at 96°C for 2 min, the DNA was denatured at 94°C for 30 s and the primers were annealed at 62°C for 30 s; the enzyme reaction was then conducted at 72°C for 2 min. This reaction cycle was repeated 18 times in a PCR Thermal Cycler MP (TaKaRa BIO, Japan), and the resulting PCR products were analyzed by electrophoresis in a 3% agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

Immunoblotting analysis

To extract glutelin A1 protein tagged with the type II collagen peptide (GluA-CII), a grain of unpolished rice was homogenized in 200 μl PBS (pH 7.5) and centrifuged at 4°C at 19,000×*g* for 3 min. The pellet was mixed with 400 μl of sample buffer (2% SDS, 20 mM Tris-HCl [pH 6.8], 6% glycerol, 0.012% bromophenol blue, and 1% 2-mercaptoethanol) and boiled for 3 min. The proteins (1/10 of the original sample, 4 μl per well) were separated by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) and transferred onto a Clear Blot Membrane-p (ATTO Corporation, Japan). The membrane was reacted with the CII-specific antibody, which had been prepared by immunizing mice with a recombinant FLAG-tagged CII protein (Hashizume et al. 2008). After incubation with peroxidase-labeled anti-mouse IgG, the immunoreactive bands were visualized with an ECL Western blot detection system (GE Healthcare Biosciences, USA) and exposing to Hyperfilm^{TM} ECL.

Results

A transgenic plant (No. 808-55) expressing the GluA-CII fusion protein at a level of 1 μg per seed (about 25 mg) was crossed with the low glutelin-content mutant, LGC-1, to examine whether the accumulation level of the GluA-CII fusion protein was increased or decreased in the presence of the *lgc1* genetic background. Based on the results obtained from genomic DNA PCR-analyses of F_3 seedlings (12 to 15 ones per F_2 plant), three F_2 plants (No. 13, 22 and 37) homozygous (+/+) for both *gluA1-coll* and *lgc1* were selected as representatives. Total proteins were extracted from F_3 seeds of these homozygous F_2 hybrid lines as well as their parent plants, LGC-1 and No. 808-55, their heterozygous F_1 and homozygous F_2 progeny, and wild-type rice (Koshihikari), and analyzed first by SDS-PAGE (Figure 2A). All of the parental and hybrid lines with the *lgc1* gene (*lgc1*+/+) showed typical LGC-1 phenotypes, i.e., a decrease in the staining intensity, or loss, of glutelin subunit bands and a marked increase in the staining intensity of prolamin bands, as compared to those of wild-type rice. On the other hand, a control F_3 hybrid line with *gluA1-coll* (*gluA1-coll*+/+) but without *lgc1*, as well as the paternal line (No. 808-55), showed a protein band pattern similar to that of wild-type rice. Such phenotypes, characterized by decreased glutelin and increased prolamin, in the LGC-1/No. 808-55 hybrid lines suggest that the *lgc1* gene was inherited by, and active in, the hybrid progeny lines. These seed proteins were then blotted on a membrane and probed with anti-CII antibody for immunoblotting analysis to evaluate the expression of the GluA-CII fusion protein in the hybrid lines. As shown in Figure 2B, the expression levels of the fusion protein in the hybrid lines were much lower than those in the paternal line (H8, No. 808-55) and a F_3 hybrid control (HC, *gluA1-coll*+/+ and *lgc1*−/−). The 65- and 32–33-kDa bands were estimated to be a precursor form of the fusion protein and the C-terminal fragment of its processed (mature) form, respectively, based on the known maturation of glutelin from a 57-kDa precursor to two 37-kDa (N-terminal) and 22-kDa (C-terminal) fragments by proteolytic processing (Sarker et al. 1986). The double bands visible at about

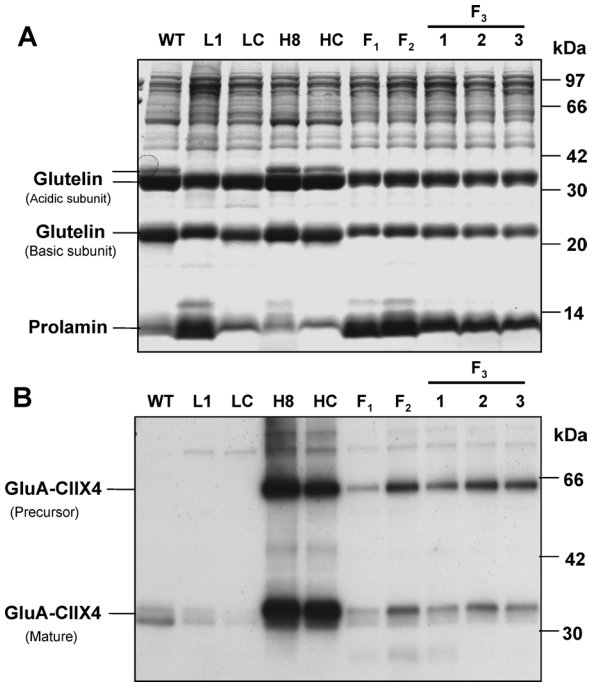

Figure 2. SDS-PAGE (A) and immunoblotting (B) analyses of total protein extracted from F_3 seeds of the homozygous F_2 hybrid line. Typical results on one F_2 hybrid line, No. 37, are shown as representatives. A seed from a wild-type plant (WT) was also analyzed as a negative control. The major endogenous protein (panel A) and immunologically stained bands expected to be precursor and mature forms of the GluA-CII fusion protein (panel B) are shown on the left, and positions of molecular standards are on the right. L1, LGC-1 (seed parent); LC, an [*lgc1*+/+ and *gluA1-coll*−/−] F_3 hybrid control; H8, a *gluA1-coll* transgenic line (No. 808-55) as a pollen parent; HC, an [*lgc1*−/− and *gluA1-coll*+/+] F_3 hybrid control; lanes 1–3, three F_3 seeds from [*lgc1*+/+ and *gluA1-coll*+/+] F_2 hybrid line (No.37).

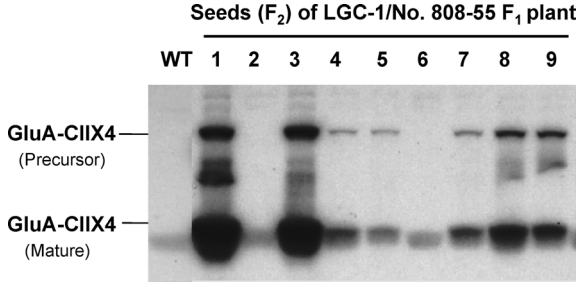

Figure 3. A typical result of immunoblotting analysis of GluA-CII fusion proteins in seeds (F_2 population) of LGC-1/No. 808-55 F_1 hybrid plants. The GluA-CII accumulation levels were classified into four groups, e.g., high (lanes 1 and 3), medium (lanes 8 and 9), low (lanes 4, 5 and 7) and none (lanes 2 and 6). A seed from a wild-type plant (WT) was also analyzed as a negative control.

Table 1. Relationship between *gluA1-coll* expression and the *lgc1* genotype in LGC-1/No. 808-55 F_2 populations

	gluA1-coll expression[1]	*lgc1*-positive[2]	*lgc1*-negative[2]
GluA-CIIX4-positive	high	0	9 (12.4%)
	middle	18 (24.7%)	0
	low	27 (37.0%)	0
GluA-CIIX4-negative	–	13 (17.8%)	6 (8.2%)

[1] Expression levels of the *gluA1-coll* transgene were estimated by immunoblotting for GluA-CIIX4 fusion proteins accumulated in the F_2 hybrid seeds (randomly selected 73 seeds), and shown as high, middle and low according to the immunostaining intensities (see Figure 3).
[2] Genotype of each F_2 seed was estimated by genomic DNA PCR analyses for the seedling from the embryo half.

32–33 kDa might be produced from a single 65-kDa precursor by slightly different proteolytic processing. Weakly stained doublet bands of about 32 kDa were observed even in the negative control lanes (WT, L1 and LC) without *gluA1-coll*, whereas no bands corresponding to the 65-kDa precursor form were detected. Such immunostaining of the 32-kDa band could be due to non-specific binding of antibodies to endogenous glutelin acidic subunits, which are the most abundant proteins in the seeds.

To analyze in more detail the effect of the *lgc1* gene on *gluA1-coll* gene expression, F_1 hybrid seedlings were cultivated and self-pollinated to obtain F_2 seeds (over 500 grains). The F_2 hybrid seeds (73 randomly selected grains) were used for both genotype and phenotype analyses. The endosperm half with the embryo was grown to an F_2 seedling and used for genomic DNA PCR analyses of both *gluA1-coll* and *lgc1*, whereas the other endosperm half was used for immunoblotting analysis of the expression of GluA-CII fusion proteins. Typical immunoblotting results are shown in Figure 3. There were large differences in the levels of fusion protein expression among the F_2 hybrid seeds. The expression levels were classified into four groups—high, medium, low and none—based on the immunostained band intensities of both the precursor and mature forms. As summarized in Table 1, all F_2 hybrid lines showing a high level of GluA-CII expression were *lgc1* negative (−/−), whereas those of *lgc1* (+/+) showed medium or low levels of expression. These results clearly indicated that *lgc1* affected *gluA1-coll* expression at the protein accumulation level.

Accordingly, *gluA1-coll* gene expression in the hybrid lines was examined at the mRNA accumulation level using RT-PCR analysis (Figure 4). Total RNAs were prepared from three genetic types of hybrid lines, i.e., [*lgc1*+/+ and *gluA1-coll*−/−] (LC as a negative control), [*lgc1*−/− and *gluA1-coll*+/+] (HC as a positive control), and [*lgc1*+/+ and *gluA1-coll*+/+] (F_4 lanes 1 to 6), and a wild-type control, Koshihikari (WT), and then mRNA that had accumulated in developing seeds was estimated by semi-quantitative RT-PCR for a GluA1-CII-encoding region of *gluA1-coll* gene transcripts, a GluA1-encoding region of the endogenous *gluA1* transcript including a 3′ UTR sequence, another GluA1-encoding region within its ORF, and ubiquitin mRNA as an endogenous standard. As shown in Figure 4

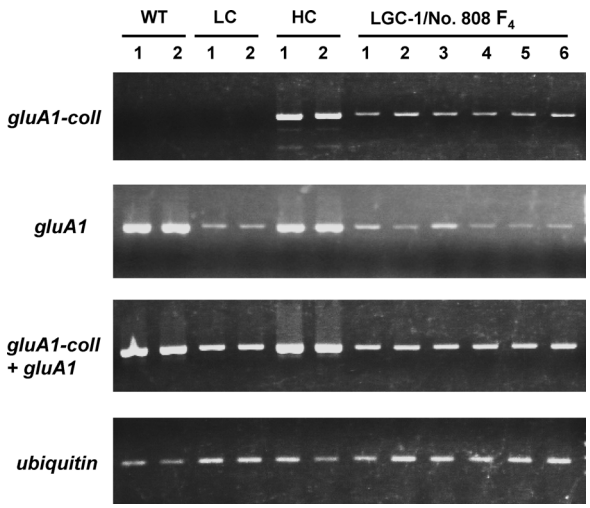

Figure 4. Semi-quantitative RT-PCR analyses for estimation of accumulated *gluA1-coll* mRNAs and endogenous *gluA1* mRNAs. Total RNAs extracted from developing F_4 seeds (15 DAF) of hybrid F_3 plants were reverse-transcribed and used as a template for PCR amplification with specific primers, as described in Materials and methods. The amplification reactions for *gluA1-coll* (440 bp), *gluA1* (1,370 bp including the 3′UTR), both *gluA1-coll* and *gluA1* (420 bp within the *gluA1*-ORF), and ubiquitin (220 bp) were done using specific primers, as described in Materials and methods. WT, Wild type; LC, an [*lgc1*+/+ and *gluA1-coll*−/−] F_4 hybrid control; HC, an [*lgc1*−/− and *gluA1-coll*+/+] F_4 hybrid control; lanes 1–6, six F_4 developing seeds from two [*lgc1*+/+ and *gluA1-coll*+/+] F_3 hybrid lines, No.37-7 (lanes 1, 2) and No. 37-8 (lanes 3–6).

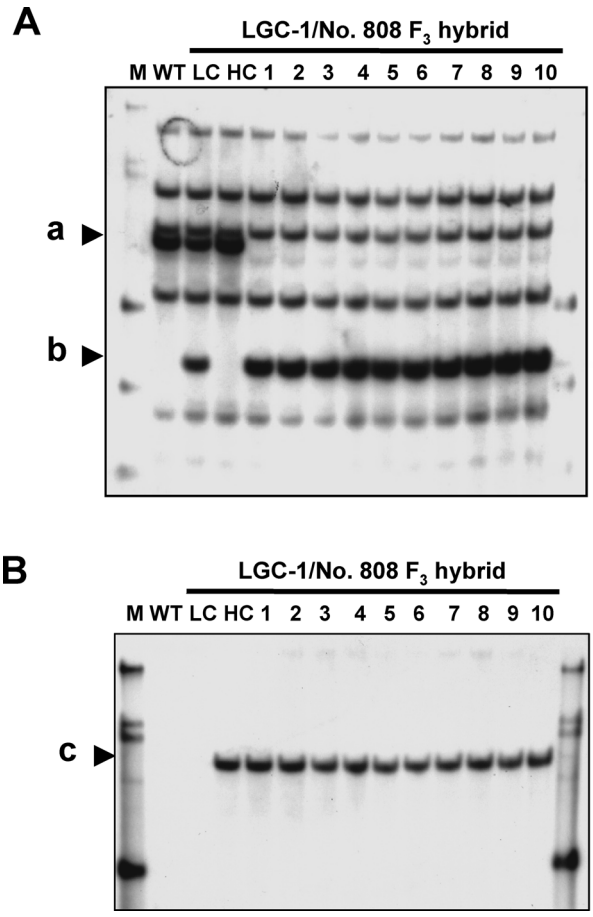

Figure 5. Genomic DNA blotting analyses of F_3 hybrid plants from homozygous F_2 hybrid lines using probes for *lgc1* (panel A) and *gluA1-coll* (panel B). The 1.5-kb DNA fragment of *lgc1* (shown as PCR-3 in Figure 1B) and the 440-bp DNA fragment of *gluA1-coll* (shown as PCR-2 in Figure 1A) were DIG-labeled and used as probes, respectively. WT, Wild type; LC, an [*lgc1*+/+ and *gluA1-coll*−/−] F_3 hybrid control; HC, an [*lgc1*−/− and *gluA1-coll*+/+] F_3 hybrid control; 1–10, [*lgc1*+/+ and *gluA1-coll*+/+] F_3 hybrid plants. (a) a band specific for the wild-type and the hybrid lines with either *lgc1* or *gluA1-coll*, (b) a band specific for the plants with *lgc1*, (c) a band specific for the plants with *gluA1-coll*.

(top panel), accumulation of mRNA transcribed from the *gluA1-coll* gene was markedly decreased in all of the six lgc*1*+/+ F_4 hybrid plants as compared to those of HC [*lgc1*−/− and *gluA1-coll*+/+] control F_4 plants. Accumulation of mRNA from the endogenous *gluA1* gene was confirmed to be decreased in the *lgc1*+/+ F_4 hybrid plants as well as LC, independently of the presence or absence of the *gluA1-coll* gene (second top panel). The endogenous *gluA1*-derived mRNA accumulation in HC was equivalent to that of the parental line of No. 808-55, Koshihikari (WT). A similar RT-PCR profile was obtained when the *gluA1*-region of mRNAs was amplified using another primer set common to both exogenous *gluA1-coll* and endogenous *gluA1* (third top panel).

In addition to genomic DNA PCR-analyses of the F_3 generation, genomic DNA blotting was also done to confirm the genotypes of the F_3 hybrid lines obtained (Figure 5). Several DNA fragments were detected in wild-type rice by hybridization with the *lgc1* probe, while one additional band was detected in the *lgc1* (+/+) F_3 hybrid lines independently of the *gluA1-coll* transgene (Figure 5A). Hybridization with a *gluA1-coll* probe showed a single band for all F_3 hybrid lines (Figure 5B). Thus, the genomic DNA blotting analyses suggested that these F_3 lines are genetically homogeneous, supporting the assumption that their parental F_2 lines are homozygous for both the *lgc1* and *gluA1-coll* genes.

Discussion

Expression of the *gluA1-coll* fusion gene was suppressed much more strongly than would have been expected from the low sequence homology between *gluA1* and *gluB4/B5*. The results shown in Figure 2 suggest that *lgc1* truly affected the expression of the *gluA1-coll* transgene in maturing seeds of the hybrid lines. However, the possible effects of genes other than *lgc1* could not be completely ruled out. In the F_2 generation, quantitative traits including total protein in rice grains would have been genetically separated, because the original varieties used as parent lines were different, i.e., No. 808-55 is derived from Koshihikari, while LGC-1 is a chemical mutant from Nihonmasari. Therefore, some differences

in the accumulation levels of GluA-CII fusion proteins of hybrid lines, especially among homozygous F_2 and F_3 lines (Figure 2B), might have been due to minor effects of some genes other than *lgc1* on the decreased accumulation of the GluA-CII fusion protein in the hybrid lines.

During seed maturation of LGC-1, the degree of mRNA accumulation of *gluA1* was reported to be about one-half at 10 DAF and about one-fifth at 16 DAF as measured by RT-PCR (Kusaba et al. 2003). In the present study, comparable suppression of endogenous *gluA1* at an mRNA accumulation level (15 DAF) was observed also in the stable hybrid lines with *lgc1*, and no remarkable differences in the mRNA accumulation were observed between the endogenous *gluA1* gene and the introduced *gluA1-coll* fusion gene (Figure 4). These results indicate that *lgc1* phenotype affected not only endogenous *gluA1* genes but also the exogenous *gluA1-coll* fusion-gene in the hybrid lines. Furthermore, marked suppression was also observed at a protein accumulation level of GluA-CII (Figure 3 and Table 1), suggesting that suppressive effect in the *gluA1-coll* gene transcription was much stronger than enhancing one in the mRNA translation due to high metabolic potential.

Kusaba et al. (2003) suggested that two dsRNA-forming regions (region A and B) might be present in *gluB4* and *δ-gluB5* of the *lgc1* genome, and showed that long (25 nt) and short (23 nt) RNA fragments were present in LGC-1 when region A was used as a probe for RNA gel blotting analysis (the *gluB4* and *gluB5* sequences are identical in region A). They further speculated that these small RNAs might be able to induce weak RNA interference against not only *gluB* but also *gluA1*, because *gluA1* shared 66.4% identify with *gluB4-δgluB5*. The sequence of *gluA1* used in the present study was compared with that of the possible dsRNA-forming region A of *gluB4-δgluB5*. As shown in Figure 6, it became obvious that perfectly matched sequences comprised 14 nucleotides at most, and it was only 23 even when one mismatch within the sequence was allowed. Therefore, it could be suggested that, in spite of such low sequence identify between the two glutelin genes (*gluA1* and *gluB4/5*), *gluA1-coll* transgene transcripts as well as endogenous *gluA1* gene products were markedly degraded, probably by *gluB4/5*-derived RNA interference. Double-stranded RNAs with shorter sequences such as 14 residues or less may function as siRNAs when a large amount of dsRNAs are produced from highly expressed genes such as seed storage protein genes. To evaluate LGC-1 as a potential host line for high expression of glutelin-fusion genes, it would be of interest to investigate the expression in LGC-1 of some transgenes fused with a mutated *gluA1* gene with further low sequence-identity to *gluB4/5* or some *gluA* genes other than *gluA1*.

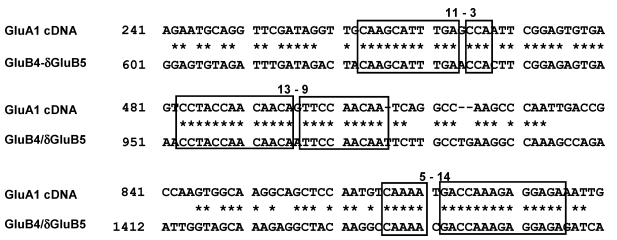

Figure 6. Sequence comparison between the possible dsRNA-forming region of *gluB4-δgluB5* (region A) (Kusaba et al. 2003) in LGC-1 and the coding region of *gluA1* (accession number: NM_001050863). In the region A of *lgc1* (accession number AB093593), the *gluB4* nucleotide sequence is identical to that of *gluB5*. Representative regions with matched sequences are boxed.

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