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The Hypocholesterolemic Activity of Transgenic Rice Seed Accumulating Lactostatin, a Bioactive Peptide Derived from Bovine Milk β-Lactoglobulin

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ABSTRACT: Lactostatin is a novel pentapeptide (IIAEK) derived from bovine milk β-lactoglobulin with greater hypocholesterolemic activity than β-sitosterol, the drug commonly used to treat hypercholesterolemia. We developed transgenic rice expressing lactostatin as a fusion protein with seed storage protein (SSP) glutelins under the control of three different endosperm-specific promoters. Lactostatin accumulated in transgenic rice seed at approximately 1.6 mg/g seeds (dry seeds) without any apparent influence on seed traits such as endogenous SSP expression levels or alterations in the intracellular structures of endosperm cells. Short-term (three day) oral administration of the glutelin fraction containing lactostatin (namely three times of 300 mg/kg body weight/day) extracted from transgenic rice seeds resulted in hypocholesterolemic activity in rats; namely, the serum low-density-lipoprotein (LDL) cholesterol level was significantly reduced accompanied by a significant increase in beneficial serum high-density-lipoprotein (HDL) cholesterol.

KEYWORDS: bioactive peptide, cholesterol, β-lactoglobulin, lifestyle disease, *Oryza sativa* L., transgenic rice

INTRODUCTION

Hypercholesterolemia is one of the most well-known lifestyle diseases and is one of the most serious risk factors causing atherosclerosis and coronary heart disease. Neglect of health such as surfeit and obesity primarily causes hypercholesterolemia, but genetic factors also cause this disease. Because patients suffering with hypercholesterolemia usually need strict, long-term dietary restrictions or pharmacotherapy, quality of life and expensive health care costs become issues. Our experimental goal is to prevent or to improve lifestyle diseases such as hypercholesterolemia through a daily diet of functional foods containing hypocholesterolemic peptides.

Rice (*Oryza sativa* L.) is an especially attractive target for delivering transgenic peptides because it is a major, staple food consumed by more than half of the world's population. The entire sequence of the rice genome is accessible, conventional genetics and breeding have a long history, and rice transformation systems are well established. Transgenic rice seed is particularly attractive as a bioreactor system because high expression levels have been achieved, and engineered peptides can be produced and sequestered exclusively in specific tissues within the rice grain. Furthermore, recombinant proteins containing bioactive peptides are stable in transgenic rice seed for several years at room temperature. Thus, we have developed a number of transgenic rice plants containing bioactive and functional peptides or proteins that can contribute to the promotion and maintenance of human health.[1−6] If staple foods such as rice, wheat and corn have any additional function for human health, we will be able to perform preventive care of lifestyle disease and improve the quality of life through the daily diet.

Lactostatin is a novel hypocholesterolemic pentapeptide (IIAEK) derived from the trypsin-digested product of bovine milk, β-lactoglobulin. Oral administration of lactostatin at a dose of 300 mg/kg body weight/day to a model hypercholesterolemic rat for 3 days significantly decreases serum low-density-lipoprotein (LDL) cholesterol.[7] Lactostatin activates expression of the cholesterol 7α-hydroxylase gene and is involved in regulating the phosphorylation of an extracellular signal-regulated kinase and intracellular Ca^{2+} concentration, resulting in the prevention or improvement of hypercholesterolemia and atherosclerosis through lactostatin-mediated LDL-cholesterol degradation.[8]

In this study, we generated transgenic rice plants expressing high levels of lactostatin as a fusion protein with seed storage protein glutelins under the control of three different endosperm-specific SSP promoters. Hypocholesterolemic activity was observed in a model hypercholesterolemic rat after short-term oral administration of the recombinant protein extracted from transgenic rice seeds.

MATERIALS AND METHODS

Plant materials. A rice (*Oryza sativa* L.) seed storage protein mutant, called a123 (cv. 'Koshihikari' background), that lacks three

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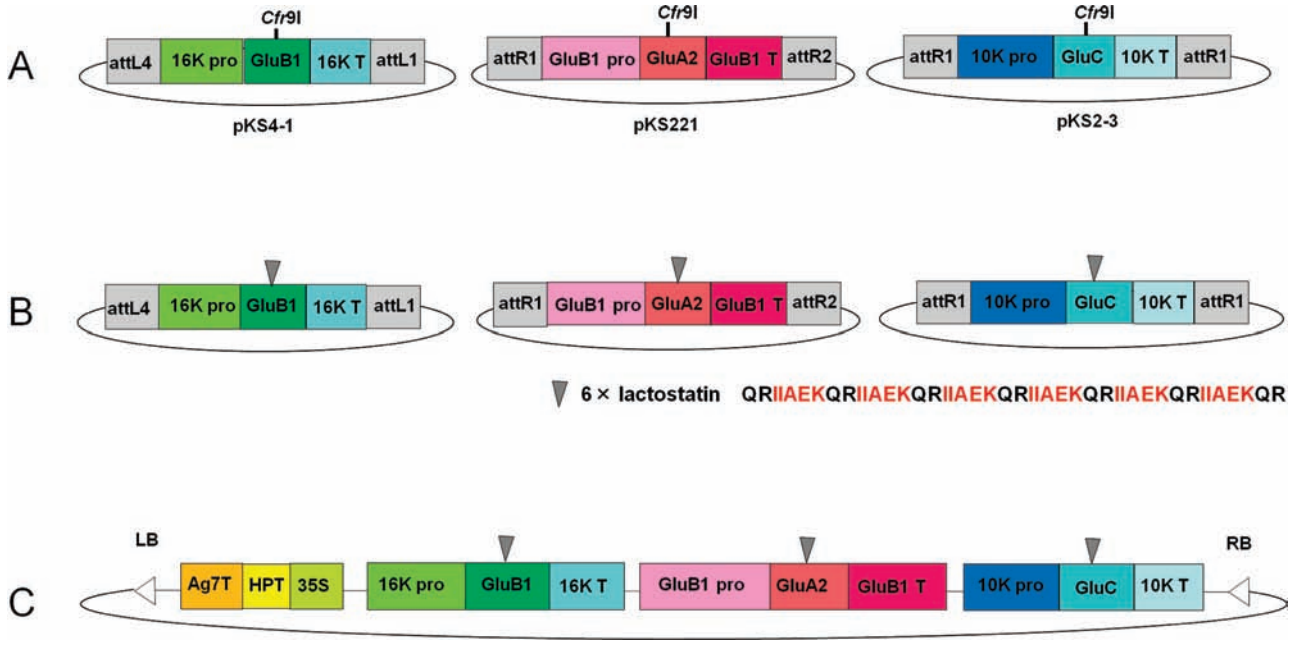

Figure 1. A schematic presentation of the binary vector constructs. (A) Individual entry clones harboring a seed-specific promoter (GluB1, 16 kDa prolamin, 10 kDa prolamin), a modified glutelin with a *Cfr*9I site (GluA2, GluB1, GluC), and a terminator derived from the rice genome (GluB1, 16 kDa prolamin, 10 kDa prolamin), respectively. (B) Entry clones harboring expression gene cassettes for glutelin-fused to 6 repeated lactostatins. (C) Binary vector construct for expression of multiple genes.

glutelins (*GluA1*, *GluA2* and *GluB4*)[9] was used as the host plant for transformation. Low glutelin mutants such as a123 and LGC1 (low glutelin content 1) are useful hosts for accruing high levels of transgenic products, because they have more vacant storage capacity for accumulating engineered products than wild type grains.[10]

Production of Transgenic Rice. Individual expression cassettes consist of the endosperm-specific promoters with their 5′ UTR (*GluB1* promoter, AY427569; 16 kDa prolamin promoter, AY427574; 10 kDa prolamin promoter, AY427572), the coding region of modified glutelin genes with their 3′UTR (*GluA2*, X05664; *GluB1*, X54314; *GluC*, AB016501) that have a *Cfr*9I site for exchange with the C-terminal variable region of the acidic subunit, and the terminators (*GluB1* terminator, X54314; 16 kDa prolamin terminator, AK287972; 10 kDa prolamin terminator, X17074), respectively. Constructs were introduced into the multiple cloning sites of each entry clone (pKS4-1, pKS221 and pKS2-3, Figure 1A). DNA fragments coding for a six tandem repeat of lactostatin (QRIIAEKQRIIAEKQRIIAEKQRIIAEKQRIIAEKQRIIAEKQR), which can be processed into active lactostatin by trypsin digestion, were prepared using optimized codons preferentially employed for the translation of several rice seed storage protein genes. A dipeptide QR spacer between each IIAEK was introduced to facilitate release of IIAEK more efficiently by trypsin digestion than the tandem repeat of IIAEK without a spacer.[11] This fragment was inserted into the *Cfr*9I site of a modified glutelin gene cassette (Figure 1B). Subsequently, these gene cassettes were introduced into p35S HPT Ag7 43 GW by the MultiSite Gateway LR clonase reaction (Invitrogen, http://www.invitrogen.com/site/us/en/home.html) as described previously[3] (Figure 1C). We previously reported that a binary vector harboring multiple gene expression cassettes, which consist of different promoter, coding region containing bioactive peptide sequence and terminator, leads to higher accumulation of bioactive peptide when compared with a single gene expression cassette.[3] Furthermore, we confirmed that length of 6 × IIAEKQR was better for stable accumulation of lactostatin without perturbation in rice seed cells.[3] A binary vector plasmid harboring three gene expression cassettes was introduced into the a123 genome via *Agrobacterium*-mediated transformation.

DNA Extraction and Southern Blot Analysis. Genomic DNA was extracted from 1.0 g of young leaves with a CTAB method.[12] Five micrograms of DNA was digested with *Hin*dIII or *Xba*I, fractionated by electrophoresis on a 0.8% agarose gel and transferred to a Hybond-N+ nylon membrane (GE Healthcare, http://www.gelifesciences.com/aptrix/upp01077.nsf/content/na_homepage). Southern hybridization and signal detection were carried out using the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare). The probe was prepared from purified PCR products of the *HPT* coding region (forward primer, 5′-gcgacgtctgtcgagaagtttctg-3′ and reverse primer, 5′-ttcggtttcaggcaggtcttgc-3′).

Protein Extraction and Immunoblot Analysis. Mature seeds from the transgenic rice line were harvested. Each grain was ground into a fine powder with a Multibeads Shocker (YASUI KIKAI, http://www.yasuikikai.co.jp/company/e_index.html). For total protein extraction, 500 μL of extraction buffer [50 mM Tris-HCl, 8 M Urea, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 1% Bromophenol Blue (BPB)] was added to the seed powder and vortexed for more than 1 h at room temperature. The mixture was centrifuged at 12000*g* for 20 min at room temperature, and the crude soluble protein sample was decanted into a new tube. Two microliters of total protein solution was subjected to immunoblot analysis after electrophoresis on 12% SDS−PAGE for quantification of transgenic products. After electrophoresis on SDS−PAGE, proteins were blotted onto Immobilon-P PVDF transfer membrane (Millipore, http://www.millipore.com/catalogue/item/ipvh07850). Membrane was reacted with primary antibody (1:10000 dilution) at 4 °C for 16 h after blocking by 5% skim milk for 1 h, followed by secondary anti-rabbit IgG conjugated HRP antibody at a 1:10000 dilution for 3 h. Signals were detected by ECL-Western blotting detection reagent (GE Healthcare, http://www.gelifesciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&moduleid=46853).

For relative comparison of lactostatin accumulation, equal amounts of the protein extracts were spotted onto a nitrocellulose membrane (Whatman, Dassel, Germany). The lactostatin in each dot was detected immunologically with anti-IIAEKQR, and quantified with NIH ImageJ (National Institutes of Health, ver. 1.41, http://www.nih.gov/).

Extraction of the glutelin fraction was performed as described previously.[10] Briefly, glutelins were extracted with 1% lactic acid from a fine seed powder after removal of the albumin and globulin fraction with a saline solution (10 mM Tris-HCl pH 7.5, 0.5 M NaCl), followed by removal of prolamins with a 60% *n*-propanol solution. The resulting glutelin fraction was used for the animal feeding study.

Transmission Electron Microscopy (TEM). Immature seeds (15−20 DAF) were fixed overnight at 4 °C in 4% paraformaldehyde, 0.1% glutaraldehyde buffered at pH 7.2 with 20 mM PIPES. After washing with PIPES buffer, the samples were dehydrated in a series of ethanol concentrations and embedded in LR White resin (ProSciTech, http://www.proscitech.com.au/cataloguex/search.asp). Ultrathin sections were cut with a glass knife using an ultramicrotome (MT2-B, Sorvall, http://www.thermoscientific.com/wps/portal/ts/) and mounted on copper grids. The section samples were reacted with primary anti-IIAEKQR or anti-GluA antibody (1:500 dilution) followed by the secondary 15 nm gold-labeled goat anti-rabbit IgG Fc SP (EY Laboratories Inc., http://www.eylabs.com/) at a 1:200 dilution.

Animals and Diet. Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) were used for this animal study. Room temperature was maintained at 22 ± 2 °C with a 12 h cycle of light (8:00−20:00) and dark. Approval from the Gifu University Animal Care and Use Committee was given for our animal experiments. All rats were housed individually in metal cages and were allowed free access to food and water. After acclimation to a commercial nonpurified diet (MF, Oriental Yeast, Osaka, Japan) for 3 days, 4 week old rats weighing about 90 g were divided into 3 groups of 7 rats each on the basis of body weight. Each group had free access to the high cholesterol diet containing casein during the experimental period. The composition of the casein diet is as follows (%): casein, 20; lard, 5.0; corn oil, 1.0; cellulose, 5.0; AIN[93] mineral mixture, 3.5; AIN[93] vitamin mixture, 1.0; cholesterol, 1.0; sodium cholate, 0.25; choline chloride, 0.2; sucrose, 21.02; and starch, 42.03. Purified glutelin fractions from either the non-transgenic (a123-glutelin) or transgenic rice (lactostatin-glutelin) were homogenized in distilled water. Glutelin fractions containing an effective dose of lactostatin (300 mg/kg body weight/day) were orally administered twice every day (0800 and 1500, namely 2 × 150 mg in total 300 mg/kg body weight/day) for 3 days. Same amounts of distilled water and suspension of glutelin fractions derived from non-transgenic were also orally administrated for 3 days. On day 4, after 4 h food deprivation, the rats were anesthetized with diethyl ether and killed. Blood was collected by cardiac puncture, and the liver was removed.

Serum and Liver Lipid Analyses. Serum and liver lipids were determined by a previously described method.[7]

RESULTS AND DISCUSSION

Characterization of Transgenic Rice. Nineteen independent transgenic rice lines were generated by *Agrobacterium*-mediated transformation. Expression levels of lactostatin in dry seed for individual transgenic lines were examined by immunoblot analysis using an anti-IIAEKQR antibody. Nine transgenic rice lines accumulated lactostatin in mature seeds, which was derived from expression of the introduced three gene cassettes as shown in Figures 2A and 2B. In order to evaluate expression levels from the individual gene cassettes, four kinds of antibodies (anti-IIAEKQR, anti-GluA, anti-GluB and anti-GluC antibodies) were used. In the nontransformed control (a123), endogenous GluB and GluC were detected by immunoblot analysis when probed

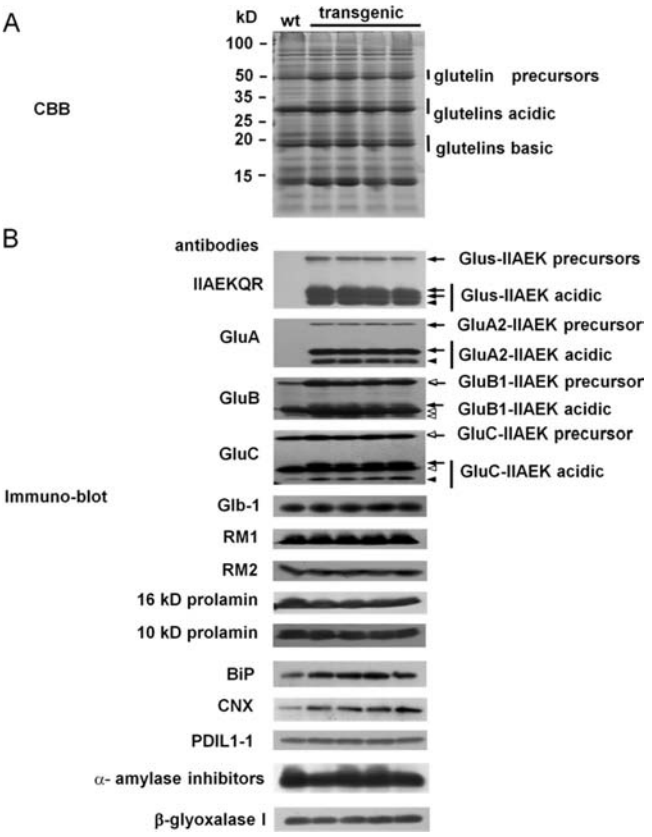

Figure 2. SDS−PAGE (A) and immunoblot analyses (B) of transgenic rice seed proteins. The accumulation of 6 tandem repeats of lactostatin (IIAEKQR), glutelins (GluA and GluC), 26 kDa globulin (Glb-1), prolamins (RM1, RM2, 10 kDa prolamin and 16 kDa prolamin), ER chaperone (BiP, CNX and PDIL1-1) proteins, and major rice seed allergens (α-amylase inhibitor and β-glyoxalase I) was investigated using specific antibodies. Arrows indicate the position of transgenic products derived from the introduced three gene cassettes, respectively. Solid arrowheads indicate the estimated degradation products derived from introduced GluA-fused lactostatin and GluC-fused lactostatin. Hollow arrowheads are endogenous glutelin (GluB or GluC) protein; wt, wild type.

with anti-GluB and anti-GluC antibodies, whereas no cross-reactivity with the other antibodies was observed. When the anti-IIAEKQR antibody was used for immunoblot analysis, one major glutelin precursor signal and three major glutelin acidic subunit signals were detected in transgenic rice seed. On the other hand, the anti-GluA antibody reacted with glutelin precursor and acidic subunit derived from GluA2-fused lactostatin. Moreover, anti-GluB and anti-GluC antibodies also reacted with GluB-fused lactostatin and GluC-fused lactostatin, in addition to endogenous GluB and GluC precursor and acidic subunit, respectively (Figure 2B). When anti-GluA or anti-GluC anitibodies were used, unexpected smaller size signals than native acidic subunits were also detected in transgenic seeds, although the majority of GluA2- and GluC-fused lactostatin accumulated as the intact form. This result suggests that some parts of the fusion proteins may be subjected to degradation due to instability. In contrast, no degradation of GluB1-fused lactostatin was detected.

We investigated the effect of transgenic products on expression of the other SSPs [26 kDa globulin (Glb-1), 13 kDa Cys-rich

prolamin (RM1) and 13 kDa Cys-poor prolamin (RM2), 16 kDa prolamin and 10 kDa prolamin], chaperone proteins [binding protein (BiP1), calnexin (CNX), protein disulfide isomerase-like 1-1 (PDIL1-1)] and rice seed allergen proteins (α-amylase inhibitor and β-glyoxalase I). As shown in Figure 2B, accumulation levels of most SSPs in transgenic rice seed were not affected by high expression of modified glutelins containing lactostatin when compared to wild type. On the other hand, the levels of two kinds of chaperone proteins, BiP1 and CNX, were 1.7- and 3.6-fold enhanced in transgenic rice seed, respectively (Figure 2). These enhanced levels may be related to heavy loading of expressed products in the ER lumen.

The 14−16 kDa α-amylase inhibitor, 33 kDa β-glyoxalase-I and 26 kDa Glb-1 are major rice allergens.[13−15] In transgenic rice seed expressing lactostatin, there was no detectable effect on the expression of these major allergen proteins (Figure 2B).

Taken together, these results indicate that accumulation of inherent seed proteins during seed development was not perturbed irrespective of the high accumulation level of modified glutelins containing lactostatin. The chimeric glutelins containing the 6 × lactostatin sequence are synthesized and effectively deposited like native glutelins in maturing endosperm cells.

Both BiP1 and CNX are responsible for protein folding in the ER lumen at an early stage of protein maturation, leading to stable accumulation of endogenous SSPs or high accumulation of exogenous recombinant protein in rice seeds.[16,17] Furthermore, BiP1 and CNX act as sensing signals to assess whether the present state of secretory proteins in the ER lumen is normal or abnormal. Especially too small or too large amounts of BiP1 result in ER stress in the cell.[17,18] We have recently demonstrated that higher concentrations of BiP and CNX are associated with higher levels of seed storage protein production by alleviating ER stress caused by the high production of SSPs.[17] That is, slight enhancement of BiP1 and CNX levels observed in this transgenic seed may contribute to greater accumulation of glutelin-fused lactostatin through efficient refolding as well as stable accumulation of endogenous SSPs.

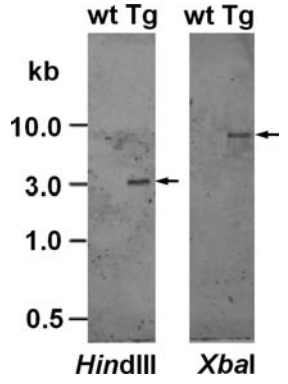

Figure 3. Southern blot analysis. Rice genomic DNA was digested by *Hin*dIII (left panel) and *Xba*I (right panel). The HPT coding region was used as a probe. Arrows indicate the hybridization signal; wt, wild type; Tg, transgenic.

The transgenic line accumulating the highest level of lactostatin in seed was advanced to the next generation, and its homozygous line was selected from T2 progeny (Figure 2). Integration of T-DNA into the rice genome of the homozygous transgenic line and its copy number were examined by Southern blot analysis using the hygromycin phosphotransferase (HPT) coding region as a probe. When the probe was hybridized to genomic DNA digested with *Hin*dIII and *Xba*I, a single copy of the transgene was found to be introduced into the transgenic rice genome (Figure 3).

The subcellular localization of transgene products in endosperm cells was examined by immuno-cytochemical electron microscopy using the antibody against lactostatin (Figure 4C). Glutelins are mainly deposited in irregularly shaped protein bodies II (PB-II) as protein storage vacuoles of wild type endosperm cells (Figure 4A). In transgenic rice seeds expressing lactostatin, anti-IIAEKQR labeled-immunogold particles were exclusively observed in PB-II but no signal was detected in wild type rice seed cells (Figures 4C and 4D). The specificity of this intracellular localization was confirmed by localization of glutelin using the anti-GluA antibody (Figure 4B). It is important to note that there is little difference in the subcellular structures of endosperm cells between non-transgenic and transgenic rice in this study. These observations suggest that the modified glutelins containing lactostatin were normally trafficked into PB-II in a manner similar to the native glutelins (Figure 4).

Accumulation levels of transgene products were quantified by dot blot immunoanalysis using the anti-IIAEKQR antibody. First, it is essential to ensure that there are no differences in the reactivity to the anti-IIAEKQR antibody among GluA2-fused lactostatin, the GluB1-fused lactostatin and the GluC-fused lactostatin. Therefore, the three fusion proteins were individually produced in an *Escherichia coli* expression system and then subjected to immuno-dot blot analysis. As shown in Figure 5A, reactivity to the anti-IIAEKQR antibody was similar among the three fusion proteins. Then, in order to examine lactostatin levels in individual transgenic rice seeds, a dot blot analysis was carried out using anti-IIAEKQR antibody and the purified GluA2-fused

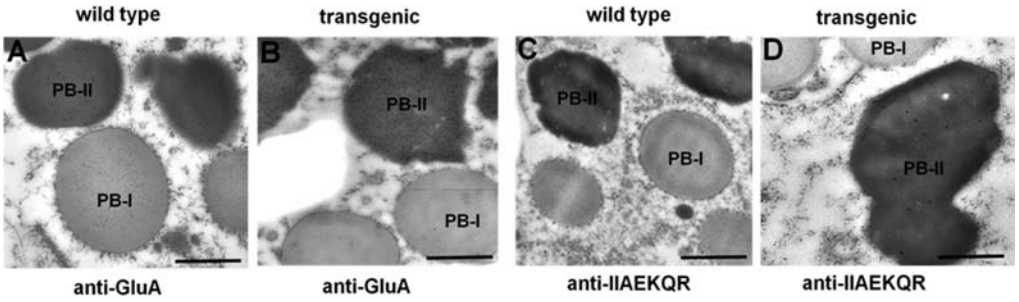

Figure 4. Localization of transgene products in transgenic rice seed cells. (A) Wild type endosperm cells reacted with anti-GluA antibody. (B) Transgenic rice endosperm cells reacted with anti-GluA antibody. (C) Wild type endosperm cells reacted with anti-IIAEKQR antibody. (D) Transgenic rice endosperm cells reacted with anti-IIAEKQR antibody. Bar = 1 μm.

lactostatin recombinant protein as a standard. Lactostatin levels were subsequently quantified with the NIH ImageJ program package (Figure 5B). The accumulation levels of lactostatin as a fusion protein with glutelin acidic subunit in the highest expressing transgenic line were estimated to be approximately 16.1 mg/g seeds on average in T2 homozygous seeds (Figure 5C). Thus, the lactstatin level was calculated to be about 1.6 mg/g seed, because six lactostatin tandem repeats (30 amino acids) account for approximately 10% of the mass of glutelin acidic subunit-fused lactostatin [GluA2-fused lactostatin acidic subunit (298 amino acids), GluB1-fused lactostatin acidic subunit (296 amino acids), or GluC-fused lactostatin acidic subunit (304 amino acids)].

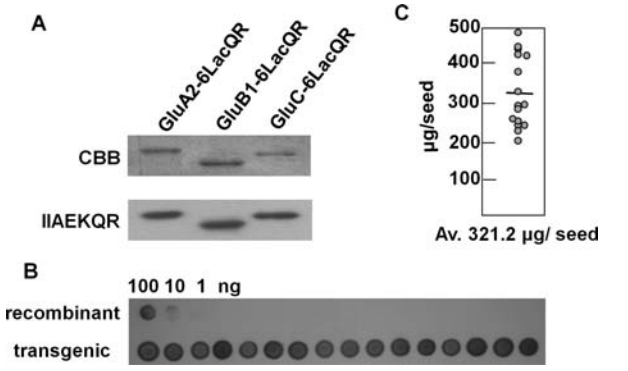

Figure 5. Dot immunoblot analysis of glutelin-lactostatin fusion proteins in transgenic rice seeds. (A) A comparison of reactivity of anti-IIAEKQR antibody against recombinant GluA1-, GluB2 and GluC-fused lactostatin, indicating that little difference was observed among these recombinant proteins. (B) Protein extracted from homozygous transgenic seeds with the highest accumulation of transgenic products was spotted onto a nitrocellulose membrane. The fusion protein level in each dot was estimated by comparison with 10-fold sequentially diluted standards of glutelin-lactostatin protein purified from *E. coli*. (C) Estimation of lactostatin accumulation levels in transgenic rice seed. The horizontal bar indicates the average accumulation level of lactostatin in transgenic rice seed.

Hypocholesterolemic Activity of Lactostatin-Fused Glutelin after Oral Administration to Rats. To determine the hypocholesterolemic activity of recombinant six tandem repeats of lactostatin fused with glutelin (lactostatin-glutelin) in transgenic rice seed, we isolated the crude glutelin fraction that contained both endogenous glutelins and lactostatin fusion-type glutelins from mature transgenic rice seeds. The glutelin fraction containing an estimated effective dose of lactostatin (300 mg/kg body weight/day) was orally administered to rats that were previously fed a high cholesterol containing diet. Identical amounts of distilled water (control-1) or a glutelin fraction (a123-glutelin as control-2) extracted from non-transgenic rice seeds were also orally administered to the respective experimental group.

No significant differences were observed in body weight, liver weight and food intake among the water-fed rats (control-1), the non-transgenic rice glutelin fraction (a123-glutelin, control-2) fed animals or the transgenic rice glutelin fraction (lactostatin-glutelin) fed animals during the oral administration experiment (Table 1). When the glutelin fraction (lactostatin-glutelin) from transgenic rice seeds was orally administered, the total serum cholesterol level was significantly reduced as compared with that of non-transgenic rice seeds (a123-glutelin, control-2) (Table 1). It was notable that LDL-cholesterol and the atherogenic index were significantly decreased after oral administration of lactostatin-glutelin when compared with that of the control-1 (water) or control-2 (a123-glutelin). Interestingly, high-density-lipoprotein (HDL) cholesterol levels were significantly increased after oral administration of lactostatin-glutelin compared with that of the control-1 and -2 (Table 1). The lower level of total serum cholesterol by oral administration of modified glutelins containing lactostatin (lactostatin-glutelin) was mainly attributed to the decrease in LDL-cholesterol. Liver lipids and cholesterol concentrations tended to be lower in the lactostatin-glutelin group compared with the control-1 (water). We speculate that an a123-glutelin-feeding itself tends to decrease the level of LDL+VLDL-cholesterol accompanied by a slight increase in HDL-cholesterol. Thus, a non-transgenic glutelin feeding is also beneficial for cholesterol metabolism. These results suggest that the six tandem repeats of lactostatin inserted in the C-terminal region of the glutelin acidic subunit are digested by trypsin in the

Table 1. Effects of Oral Administration of al23-Glutelin (Wild Type) or Lactostatin-Glutelin (Transgenic) on Body and Liver Weights, Food Intake, Serum and Liver Lipids in Rats Fed a High Cholesterol Containing Diet[a]

	group		
	water (control-1)	al23-glutelin (control-2)	lactostatin-glutelin (transgenic)
body wt gain, g/3 days	2.5 ± 0.6 a	3.3 ± 0.4 a	3.5 ± 0.8 a
liver wt, g/100 g body wt	4.65 ± 0.09 a	4.64 ± 0.15 a	4.74 ± 0.13 a
food intake, day 3, g/day	13.5 ± 0.4 a	12.5 ± 0.4 a	12.6 ± 0.4 a
serum, mmol/L			
total cholesterol	9.93 ± 0.35 b	9.27 ± 0.21 b	8.27 ± 0.22 a
HDL cholesterol (*a*)	0.80 ± 0.02 a	0.88 ± 0.04 ab	0.92 ± 0.04 b
LDL + VLDL cholesterol (*b*)	9.13 ± 0.33 b	8.39 ± 0.23 b	7.35 ± 0.22 a
atherogenic index (*b*)/(*a*)	11.37 ± 0.28 c	9.75 ± 0.72 b	8.04 ± 0.42 a
liver, μmol/g liver			
total lipid	150.7 ± 5.3 a	144.5 ± 4.8 a	136.9 ± 4.2 a
cholesterol	51.2 ± 1.2 a	47.3 ± 2.6 a	47.5 ± 1.4 a

[a] Sample dissolved in distilled water was administered orally twice a day at 8:00 a.m. and 3:00 p.m. for 3 days using a zonde. Feeding period was 3 days, and fasting period was last 4 h. Values are means ± SEM, $n = 7$. Within a row, means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test. Values were calculated as follows: LDL + VLDL-cholesterol = total cholesterol − HDL cholesterol.

small intestine and the released lactostatin peptides act as a hypocholesterolemic effecter in rat intestines. Our preliminary results may suggest that lactostatin acts as the ATP binding cassette transporter A1 related to the intestinal cholesterol absorption without decrease of micellar solubility of cholesterol related to the inhibitory effects of cholesterol absorption by beta-sitosterol. As the inhibitory action mechanism of the intestinal cholesterol absorption is different between lactostatin and beta-sitosterol, the additive effect of both lactostatin and beta-sitosterol is expected in the case of a simultaneous administration. Furthermore, beta-sitosterol can especially apply for oily foodstuff as fat-soluble material, while a water-soluble lactostatin can apply to drink. Taken together, hypochlesterolemic activity of transgenic rice seed accumulating lactostatin was clearly demonstrated in animal study. Our results suggested that the transgenic rice evaluated in this study will be able to serve as a potential functional food with a preventive effect for lifestyle disease patients such as those with hypercholesterolemia.

At present, our study is still at an experimental stage using a model animal. Thus, many kinds of safety evaluations such as out-crossing with transgenic pollen in the field and chronic toxicity or allergenicity by oral feeling will be required as next step before practical use of transgenic rice accumulating lactostatin. Furthermore, arguments about production system, delivery system and monitor system of transgenic crops with human health promoting function in the marketplace are also necessary and then common rules about these issues have to be established worldwide in cooperation with various fields of related people including scientists, medical doctors, economists and farmers.

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ABBREVIATIONS USED

CBB, Coomassie brilliant blue; CTAB, cetyltrimethylammonium bromide; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GM crop, genetically modified crop

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