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ORIGINAL PAPER

High-level production of lactostatin, a hypocholesterolemic peptide, in transgenic rice using soybean A1aB1b as carrier

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Abstract Hypercholesterolemia, a form of cardiovascular disease, is one of the leading causes of deaths worldwide. Lactostatin (Ile-Ile-Ala-Glu-Lys), derived from β-lactoglobulin in cow's milk, is a bioactive peptide with hypocholesterolemic activity higher than sitosterol, a known anti-hypercholesterolemic drug. Here, we successfully developed a transgenic rice accumulating a much higher level of lactostatin by inserting 29 IIAEK sequences into the structurally flexible (nonconserved) regions of soybean seed storage protein, A1aB1b, and introducing it into LGC-1 (low glutelin content mutant 1) as host variety. A1aB1b containing 29 lactostatins was expressed in the endosperm of rice seed cells by using seed specific promoters and sorted into novel compartments distinct from normal PB-I (ER-derived protein body) and PB-II (protein storage vacuoles). Transgenic rice seeds accumulated approximately 2 mg of lactostatins/g of dry seeds, which is relatively high compared with previous reports. Our findings suggest that the introduction of a high copy number of bioactive peptide into seed storage proteins as carrier is one of the effective means in producing higher amounts of bioactive peptides in rice.

Keywords Bioactive peptide · Rice · Seed storage protein · Lactostatin

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Introduction

Cardiovascular diseases (CVD) remain to be the major cause of deaths representing about 30 % of all deaths worldwide (Gaziano et al. 2010; Chobanian et al. 2003). Although cardiovascular mortality rates have declined in many highly-developed countries in the recent decades, cardiovascular deaths and diseases have increased at a fast rate in underdeveloped and developing countries. Among other factors, CVD is primarily correlated with hypercholesterolemia. Hypercholesterolemia, in turn, is brought about by obesity and unhealthy lifestyle, including overeating

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and lack of exercise. It can also be due to a genetic defect and could be aggravated by excessive alcohol drinking and/or cigarette smoking (Bridget and Fuster 2010). Therapy usually involves expensive continuous medication and strict and long-term dietary restrictions. We have long been involved in the development of functional foods with enhanced nutraceutical/bioactive properties that may help in preventing lifestyle diseases (Tandang-Silvas et al. 2011; Maruyama et al. 2011).

Lactostatin (IIAEK) is a novel hypocholesterolemic peptide, derived from cow's milk β-lactoglobulin, which acts to decrease serum low-density-lipoprotein and has greater hypocholesterolemic activity than that of β-sitosterol, a known drug for the treatment of hypercholesterolemia (Morikawa et al. 2007). Moreover, it was reported that lactostatin-mediated cholesterol degradation involves a new regulatory pathway in the calcium-channel-related MAPK signaling pathway (Morikawa et al. 2007). Therefore, lactostatin can be considered as a promising molecule for preventing hypercholesterolemia and atherosclerosis.

Glycinin, a major seed storage protein of soybean, is composed of five subunits. Previously, we determined the three-dimensional structure of A1aB1b, one of the glycinin subunits, and found that it forms trimers (pro-form) in its asymmetric unit (Adachi et al. 2001). The two trimers eventually form a hexamer (mature-form) after post-translational processing in seeds. The structure of the monomer is characterized by a core domain consisting of two jelly-roll beta-barrels, and two extended helix domains. Interspersed along the length of the A1aB1b monomer are highly flexible regions (named regions I through V), non-conserved among the other 11S globulins.

There have not been many reports about the large-scale production of small peptides in *Escherichia coli* and other expression systems, because of the difficulties in detection or their low expression. We have already reported that multimers of lactostatin repeats [(IIAEKIIAEKIIAEKIIAEK)n] can be introduced into A1aB1b flexible regions and that A1aB1b containing 34mer of lactostatin can be produced in significantly higher level in *E. coli* (Prak et al. 2006). Furthermore, lactostatin can be easily digested by trypsin, and a high yield of IIAEK can be obtained after purification. On the other hand, *E. coli* with plasmid encoding tandem lactostatin multimers with no carrier protein showed only a 2 % expression level of the peptide relative to total protein (Prak et al. 2005). These experiments suggest that A1aB1b might be a suitable carrier for bioactive peptide production in plant seeds.

Rice is an ideal platform for high-level expression and large-scale production of recombinant proteins in plant (Yang et al. 2008). A certain protein could be designed to be specifically produced in rice seeds. Moreover, rice has greater biomass unlike other seeds, can be easily processed after harvest, and is consumed by more than half of the global population, making it an ideal system for producing and delivering the bioactive peptides. Low glutelin content rice variety (*Oryza sativa*, LGC-1) has a 3.5-kb deletion between two highly similar glutelin genes, glutelin B4 and B5 (Kusaba et al. 2003), and has been observed to enhance the accumulation of any foreign product (Tada et al. 2003). Here, we report the accumulation of A1aB1b containing 29 lactostatins in transgenic rice seeds of LGC-1. In addition, accumulation levels and localization of A1aB1b containing lactostatins in seeds of transgenic rice were examined.

Materials and methods

Construction and introduction of transgenes into rice

The cDNA for A1aB1b containing 29 lactostatin sequences (A1aB1b29Lac, Fig. 1) was prepared from pETA1aB1b-34IIAEK, the expression plasmid for A1aB1b containing 34 lactostatin sequences, constructed previously (Prak and Utsumi 2009). The construction of the final binary vector was performed using the Multi-Site Gateway System (Wakasa et al. 2006). Initially, three entry clones/gene cassettes; (1) A1aB1b29Lac under 10-kDa prolamin promoter (AY427572) and terminator (X17074), (2) A1aB1b29Lac under glutelinB4 promoter (AY427571) and glutelinB1 terminator (X54314), and prolamin RNAi (45 bp region × 2) under 13-kDa prolamin promoter and terminator (Kuroda et al. 2010) were prepared (Fig. 2a) by inserting into vectors pKS4-1MCS, pKS221MCS, and pKS2-3MCS, respectively. All the three entry clones were introduced into the binary vector p35SHPTAg7-43GW through MultiSite Gateway LR clonase reactions (Wakasa et al. 2006). Another construct was also made containing

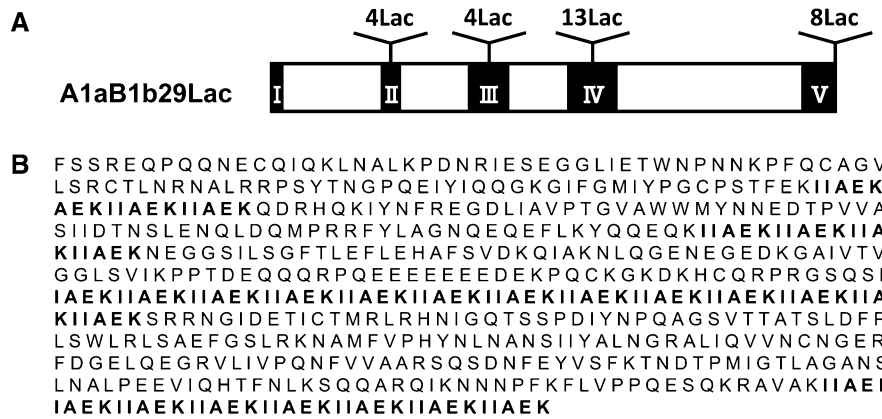

Fig. 1 Schematic structure of A1aB1b29Lac. **a** Schematic illustration of the positions of lactostatin sequence in A1aB1b29Lac gene. *I–V* indicate the structurally flexible regions of A1aB1b (Adachi et al. 2001). **b** Amino acid sequence of A1aB1b29Lac. *Bold letters* indicate the lactostatin sequences

only the wild-type A1aB1b for both prolamin and glutelin promoter-driven cassettes (A1aB1bWT) as a control (Fig. 2b). The resulting plasmids were transformed into *Agrobacterium tumefaciens* EHA105 (Hood et al. 1993) by electroporation as described previously (Goto et al. 1999).

The transformed *Agrobacterium* was then used to infect calli derived from a mature rice seed (*Oryza sativa* L. cv. LGC-1) for 3 days. Next, the calli were successively cultured in N6D selection medium (supplemented with 3 % sucrose, 0.4 % gelrite, 100 mg/L myoinositol, 300 mg/L casamino acids, 2,878 mg/L proline, 2 mg/L 2,4-D, N6 Vitamin, 500 mg/L Claforan and 25 mg/L hygromycin) for about 3 weeks, and MS regeneration medium (Murashige and Skoog 1962) supplemented with 3 % sucrose, 3 % sorbitol, 0.8 % agar, 2 g/L casamino acids, 0.2 mg/L NAA, 2 mg/L kinetin, B5 vitamin and 250 mg/L Claforan) for several weeks at 28 °C under continuous light condition (Goto et al. 1999). Transgenic plants were grown in a controlled greenhouse.

Antibodies

Antisera against lactostatin (anti-IIAEK peptide) derived from rabbit were used. Anti-rabbit IgG conjugated with alkaline phosphatase was used as secondary antibody.

Dot blot analysis

Mature seeds from independent transgenic rice lines were dehulled and individually ground into fine powder with a multi-beads shocker (Yasui Kikai, Tokyo). For total protein extraction, 20 µl of 1× SDS buffer [62.5 mM Tris–HCl (pH 6.8), 10 % v/v glycerol, 5 % v/v 2-mercaptoethanol, 2.5 % w/v SDS] was added to 1 mg seed powder and vortexed for at least 1 h at room temperature. The mixture was centrifuged at 12,000×*g* for 15 min at 4 °C, and the supernatant was then transferred into a new tube. Extracts from 8 seeds of an independent plant were pooled and blotted on nitrocellulose membrane (Whatman, Dassel, Germany). Pooled protein extract from seeds of rice (var. LGC-1) was used as negative control. The lactostatin in each dot was detected immunologically with an anti-lactostatin antibody and accumulation level was estimated densitometrically with NIH ImageJ (National Institutes of Health, Washington, DC, USA).

SDS-PAGE and western blotting

SDS-PAGE was done using 11 % polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue R-250. Western blotting was done after SDS-PAGE using a Laemmli 11 % polyacrylamide gel (Laemmli 1970). The separated proteins on gels were transferred electrophoretically to nitrocellulose membrane (0.45 µm; Schleicher and Schuell Inc., Dassel, Germany) and recombinant proteins were detected with appropriate rabbit-derived anti-sera followed by a goat anti-rabbit IgG-alkaline phosphatase conjugate (Promega, Madison, WI, USA).

Multi-stage extraction of different proteins and A1aB1b29Lac solubility

Using 40 mg of powdered seeds (n = 8) of each independent plant, different protein fractions were sequentially extracted based on their solubility to

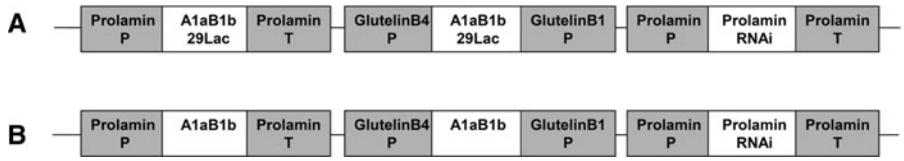

Fig. 2 Construction of expression vectors for rice transformation. Each clone harbors seed-specific promoters (from 10-kDa prolamin and glutelinB4) and terminators (10-kDa prolamin, glutelinB1) derived from rice. **a** A1aB1b29Lac, **b** A1aB1bWT. *P* and *T* indicate promoter and terminator, respectively

different buffers. Albumins and glutelins were first extracted by adding 400 µl of 10 mM Tris–HCl (pH 7.5) containing 0.5 M NaCl and vortexing for an hour and followed by centrifugation at 12,000×*g* for 15 min at 4 °C to obtain the crude extract. Cysteine-poor prolamins (P1), Cysteine-rich prolamins (P2), glutelins (Glu), and remaining residue or insoluble proteins (R) were extracted with 60 % *n*-propanol, 60 % *n*-propanol containing 5 % 2-mercaptoethanol, 5 % lactic acid containing 1 mM EDTA, and 1× SDS buffer, respectively. In addition, total protein fraction was extracted from 20 mg of powdered seed by adding 400 µl of 1× SDS buffer. All samples were concentrated by tricholoacetic acid precipitation (Maniatis et al. 1982) and 10 µl of each sample was subjected to 11 % gel SDS-PAGE (Laemmli 1970) and western blot analysis.

Transmission electron microscopy

Developing rice seeds (DAF 14) were cut into 1.5–2.0 mm sections and fixed for 2 h in 4 % (v/v) formaldehyde, 0.05 % (v/v) glutaraldehyde solution at 4 °C. Tissue sections were washed four times with buffer (100 mM sodium phosphate, pH 7.2), dehydrated in a graded ethanol series and embedded in LR White resin (London Resin, Basingstoke, UK). Ultrathin sections (60–80 nm) were obtained with a glass knife and placed on formvar/carbon-coated grids. The sections were blocked with 1 % (w/v) BSA-PBS and then incubated for 1 h at room temperature on a drop of anti-proA1aB1b, anti-glutelin, anti-prolamin, or anti-BiP in 1 % (w/v) BSA-PBS. The sections were washed on a drop of 1 % (w/v) BSA-PBS and then incubated with goat anti-rabbit IgG conjugated to 15 nm gold particles (H + L, Auro Probe EM, Amersham) in 1 % (w/v) BSA-PBS at room temperature. After washing with PBS, sections were washed twice with distilled water. The sections were stained for 25 min with 4 % (w/v) uranyl acetate followed by incubation with 80 mM lead nitrate for 25 min. The grids were examined and photographed using a transmission electron microscope (model H-7100, Hitachi, Tokyo).

Expression and purification of A1aB1b29Lac in *Escherichia coli*

The cDNA coding for proA1aB1b29Lac was introduced with poly-histidine tag into pET21d vector (Novagen, CA) and transformed into *E. coli* AD494(DE3) (Novagen, CA). The bacteria were cultured in Luria–Bertani medium with 50 µg/ml carbenicillin and expression of the recombinant protein was induced with isopropyl β-D-1-thiogalactopyranoside (1 mM final concentration) when the culture reached an $OD_{600\ nm} = 0.6$. After induction, cultures were incubated for an additional 32 h at 20 °C. Cells were then harvested, resuspended, and sonicated in buffer A [35 mM potassium phosphate (pH 7.4), 0.4 M NaCl, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride, 1.2 µM leupeptin, 0.2 µM pepstatin A, 0.02 % (w/v) NaN_3]. Since rA1aB1b29Lac is completely insoluble, the pellet which was harvested after centrifugation was further solubilized in 8 M urea buffer at 40 °C for 2 h. The resulting soluble fraction was subjected to Ni Sepharose (GE Healthcare Life Sciences, NJ) affinity chromatography to obtain the pure recombinant proA1aB1b29Lac protein.

Results and discussion

Design of binary vector construct for A1aB1b29Lac

In this study, we did not use the flexible region I (N-terminal region of A1aB1b) for the introduction of the lactostatin sequences, because of the retention of the signal peptide of A1aB1b in the final construct for transformation in rice (Fig. 2). We have used

promoters (promoters derived from 10-kDa prolamin, 13-kDa prolamin, and glutelin B4 genes) that have been reported to exhibit significantly high level protein expression specific to the endosperm of rice seeds (Qu and Takaiwa 2004). We also used a simple binary vector construction system for the simultaneous expression of multiple genes using the MultiSite Gateway System and have successfully integrated three gene cassettes in the final vector, namely, A1aB1b29Lac (Fig. 2a). Prolamin RNAi was also integrated with the aim of reducing endogenous prolamin expression and further upregulation of A1aB1b29Lac expression due to the tendency of cells to maintain overall nitrogen content and consequently, protein content regardless of the type of proteins present in the cell (Tada et al. 2003).

Transgenic rice accumulating A1aB1b29Lac

Dot blot analysis, using A1aB1b antisera, of transgenic lines obtained has confirmed the positive accumulation of A1aB1b29Lac and A1aB1bWT in their mature seeds. In order to check for post-translational processing (i.e. cleavage of the protein into acidic and basic form) of A1aB1b29Lac, western blot analysis was done using pooled extracts of T_1 seeds transformed with A1aB1b29Lac and A1aB1bWT (Fig. 3). In the case of A1aB1bWT lines, some of the A1aB1b proteins were processed into their acidic and basic chains and some accumulated as pro-form (Fig. 3a). The result is consistent with our previous work wherein soybean glycinin was also expressed both in its pro-form and processed form upon introduction into rice (Katsube et al. 1999; Takaiwa et al. 2008). However, all 11 A1aB1b29Lac transgenic lines showed unprocessed form (pro-form) of the protein and no mature form of A1aB1b has been observed upon western blotting by anti-lactostatin (Fig. 3b). SDS-PAGE patterns of several lines of transgenic rice accumulating A1aB1b29Lac also suggest the reduction of prolamin. We found several independent lines indicating the relatively high intense bands on western blotting against lactostatin antibody, indicating that these lines accumulate A1aB1b29Lac at a higher level (lower panel of Fig. 3b, arrowhead). In parallel, prolamins seem to be reduced on SDS-PAGE in these lines (upper panel of Fig. 3b, arrow). Therefore, the accumulation level of A1aB1b29Lac might be related to the reduction of prolamins.

Solubility of A1aB1b29Lac

Seed proteins are generally classified into four categories according to their solubility (Osborne 1907): (1) albumins, which are soluble in water or dilute salt solutions and are coagulated by heat; (2) globulins, which are insoluble in pure water, but soluble in dilute salt concentrations and insoluble at high salt concentrations; (3) prolamins, which are soluble in aqueous alcohol; and (4) glutelins, which are soluble in dilute acid or bases, detergents, or dissociating (urea) or reducing (2-mercaptoethanol) agents. Prolamins can be further classified into cysteine-poor and cysteine-rich type based on the absence or presence of cysteine residues in the protein, respectively. Based on this, we performed a step-wise extraction of the four major proteins in the transgenic lines and determined at which fraction A1aB1b29Lac will be detected (Fig. 4a). Endogenous A1aB1b belongs to globulin and is soluble in aqueous salt solution. In transgenic rice seeds of A1aB1bWT, A1aB1b proteins have been detected mainly in the globulin fraction and some have been found in the glutelin fraction (Fig. 4b). On the other hand, none of the A1aB1b29Lac proteins have been detected in the globulin fraction, but all were found in the glutelin and insoluble fractions. These results indicate that the introduction of IIAEK sequence into A1aB1b changed the solubility of A1aB1b in transgenic rice seeds.

Immuno-electron microscopy and localization of A1aB1b29Lac in rice seed cells

Rice seed endosperm has two types of seed storage organelles or protein bodies (PBs). Type I protein body (PB-I) is derived from endoplasmic reticulum and accumulates prolamins, while type II protein body (PB-II) is derived from vacuole and mainly consists of glutelins and globulins. In the previous study, A1aB1b in transgenic rice were deposited into the matrix of the glutelin-containing protein body II (Katsube et al. 1999; Takaiwa et al. 2008). In transgenic rice seeds producing A1aB1b29Lac, novel compartments morphologically different from PB-I and PB-II, which were not found in untransformed rice variety (LGC-1), were observed (Fig. 5a and b). The relative frequency of PB-I in transgenic rice seeds accumulating A1aB129Lac was also low compared to that of non-transgenic rice seed (var. LGC-1). A1aB1b29Lac

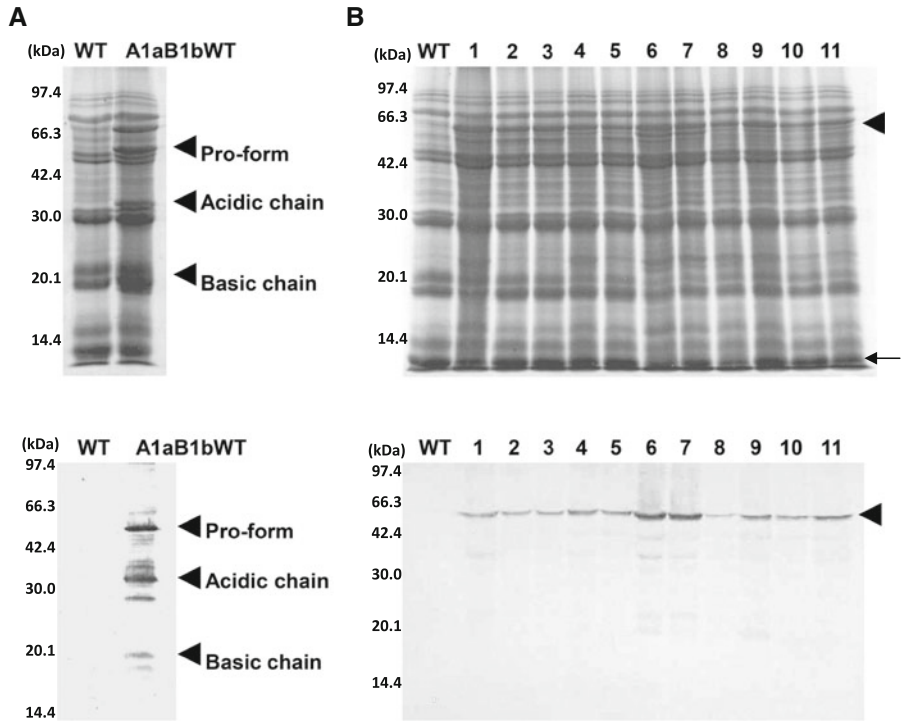

Fig. 3 SDS-PAGE and western blot detection of A1aB1b29Lac. **a** SDS-PAGE and western blot analyses of transgenic rice seed proteins of WT (LGC-1) and A1aB1bWT using anti-proA1aB1b antibody as probe. *Arrowheads* indicate either intact or processed forms of A1aB1b protein. *Upper* and *lower panels* indicate Coomassie Brilliant Blue staining and western blotting, respectively. **b** Western blot analyses of all independent transgenic lines against anti-lactostatin antibody. *Arrowheads* indicate A1aB1b29Lac. Each *lane* is taken from pooled extract of 8 seeds from an independent line. *Arrow* indicates bands corresponding to major prolamins. *Upper* and *lower panels* indicate Coomassie Brilliant Blue staining and western blotting, respectively

accumulated in these novel compartments (Fig. 5b, c). Furthermore, we tested for the localization of the chaperone protein BiP, which is a characteristic of the ER lumen and involved in folding and assembly of newly synthesized proteins, and found them also predominantly within the novel compartments (Fig. 5d). To further characterize these compartments, we also examined the localization of prolamins, a known ER (PB-I)-localized protein, and found them to be also deposited in these novel compartments (data not shown). These results suggest that the novel compartments in transgenic rice seeds accumulating A1aB1b29Lac are derived from the ER. Moreover this is consistent with the observation that A1aB1b29Lac was not processed into mature form, because it is though that the processing occurs in the vacuole (PB-II) (Fig. 3b). Recently, a model for the internal structures of PB-I in rice seed has been proposed and it suggests that molecular species of prolamins form the individual stacked layers in PB-I (Saito et al. 2012). In transgenic rice seeds accumulating A1aB1b29Lac at a higher level, expressions of some species of prolamins were probably suppressed by RNAi and, instead, A1aB1b29Lac accumulated (Fig. 3b). Therefore, the change in the protein composition in seeds accumulating A1aB1b29Lac might have led to the formation of the novel compartments in these seeds (Fig. 6).

Accumulation level of A1aB1b29Lac in rice endosperm

In order to quantify the amount of A1aB1b29Lac accumulation in the transgenic lines, we performed a dot blot analysis of the seed extracts of each of the 11 lines of transgenic rice accumulating A1aB1b29Lac and compared them with known concentrations of purified A1aB1b29Lac from *E. coli* (Fig. 6). Briefly, the recombinant A1aB1b29Lac (rA1aB1b29Lac) was extracted from inclusion bodies and purified to

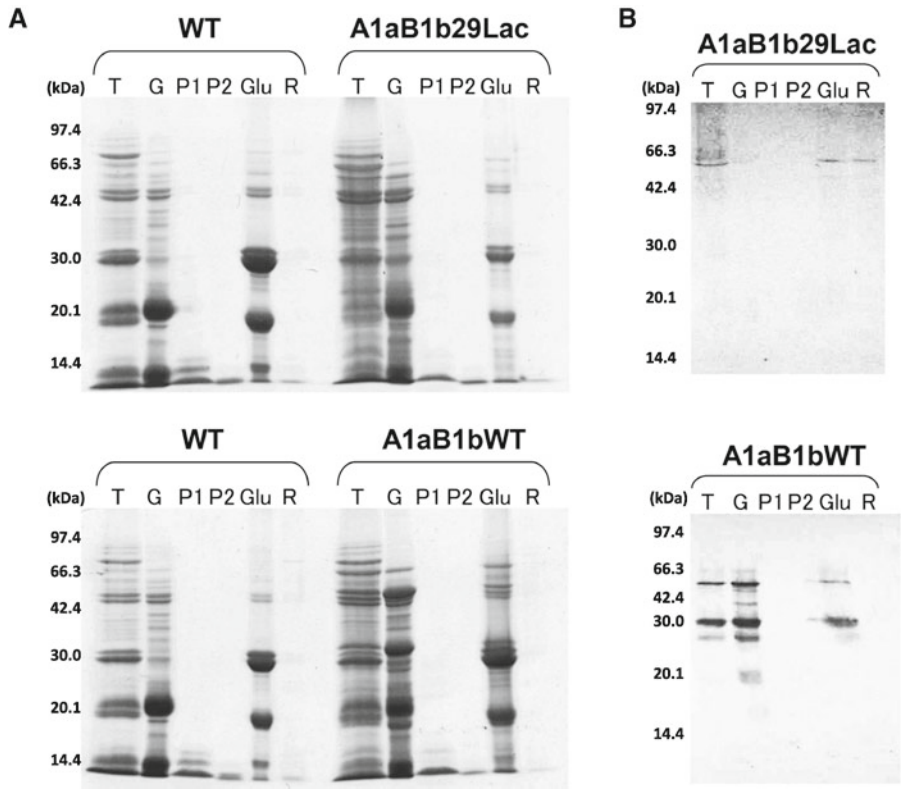

Fig. 4 Solubility of A1aB1b29Lac in the transgenic lines. **a** SDS-PAGE patterns by Coomassie Brilliant Blue staining of multi-stage extraction of different proteins [cysteine-poor prolamins (P1), cysteine-rich prolamins (P2), glutelins (Glu), and remaining residue or insoluble proteins (R)] of transgenic lines accumulating A1aB1bWT or A1aB1b29Lac. WT indicates a non-transgenic line (LGC-1). **b** Western blot assay of the extracts of transgenic lines producing A1aB1bWT and A1aB1b29Lac using anti-proA1aB1b and anti-lactostatin antibody as probe, respectively. Each *lane* is taken from pooled extract of 8 seeds from an independent line

homogeneity through Ni affinity chromatography. Varying concentrations of rA1aB1b29Lac were used to create a standard curve using optical densitometry and serve as reference for the quantification of the A1aB1b29Lac present in transgenic rice. One g of the transgenic seeds can approximately contain 1–8 mg of A1aB1b29Lac and an average of 4 mg. Since the lactostatin multimers occupy around 25 % of the total mass of A1aB1b29Lac protein and using the highest accumulating line (approximately 8 mg per g seed), the accumulation level of lactostatin in this transgenic rice is therefore as high as approximately 2 mg per 1 g of seeds. In the previous report (Wakasa et al. 2011), the lactostatin was produced by introducing 6-mer of IIAEK into three rice glutelin cassettes, resulting in the accumulation of approximately 1.6 mg of lactostatin/g of transgenic seed. Therefore, this amount in this study is relatively high compared with the yield of previous report (Wakasa et al. 2011).

Wakasa and others tried short-term (3 days) oral administration containing lactostatin (namely three times of 300 mg/kg body weight/day) extracted from transgenic rice seeds and this 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. Previously, we confirmed that lactostatin can be released from purified recombinant A1aB1b containing a high copy of lactostatin by digestion with trypsin (Prak and Utsumi 2009). Therefore, it is expected that lactostatin can also be released from A1aB1b29Lac protein extracted from transgenic rice seeds and that it would exhibit the same bioactivity in animals.

It has been established that the best way for production of bioactive peptide in transgenic plants is for the peptide to be synthesized as part of a seed

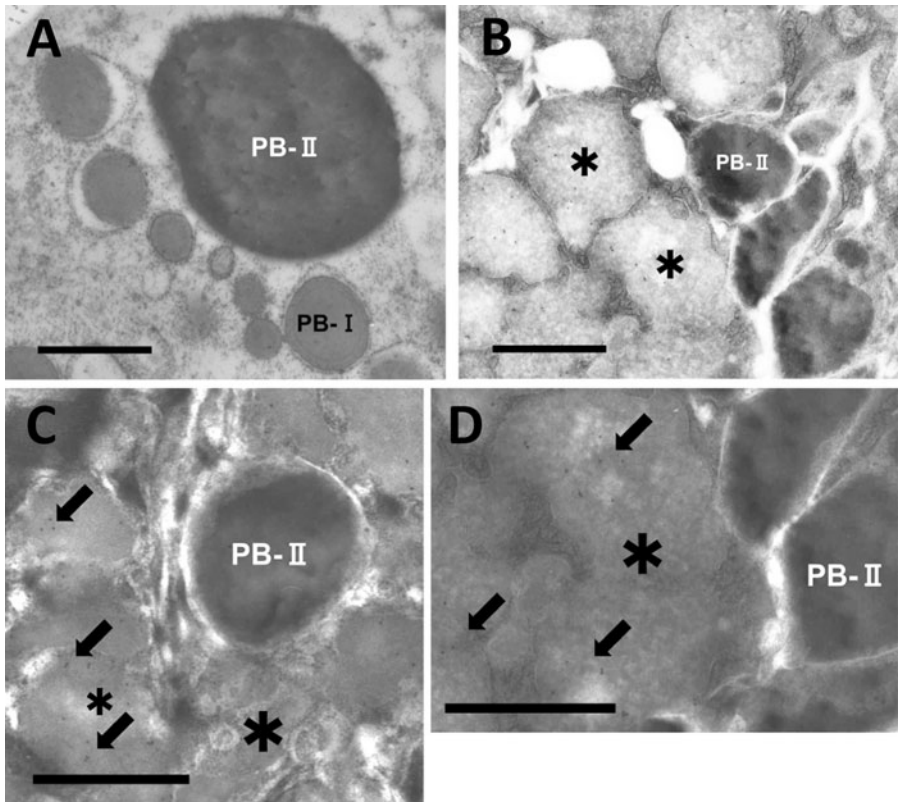

Fig. 5 Sub-cellular localization of A1aB1b29Lac in endosperm cells of rice. **a** LGC-1 WT lines. **b** Transgenic rice seeds accumulating A1aB1b29Lac detected with anti-lactostatin antibody. **c** Transgenic rice seeds accumulating A1aB1b29Lac detected with anti-proA1aB1b antibody. **d** Localization of BiP chaperone were detected using anti-BiP antibody. *Asterisks* indicate novel compartments morphologically not found in wild-type rice. *Scale bars* indicate 1 μm

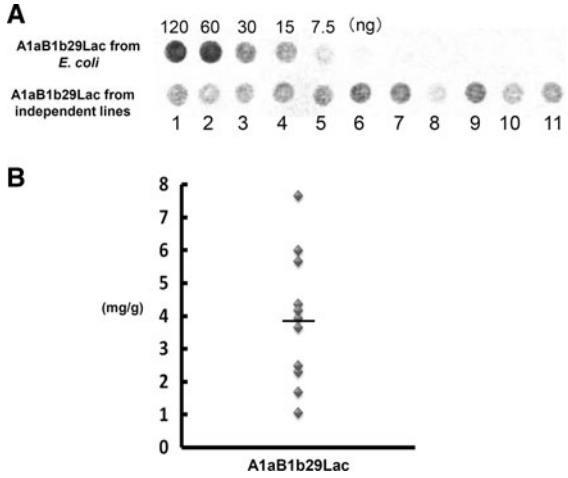

Fig. 6 Accumulation level of A1aB1b29Lac in independent transgenic lines. **a** Dot blot analysis of A1aB1b29Lac expression level compared with known concentrations of recombinant A1aB1b29Lac purified from *E. coli.* **b** Accumulation levels of A1aB1b29Lac in the 11 independent lines

storage protein (Takaiwa 2005). When a small size of bioactive peptide (less than 30 amino acids) was directly synthesized in transgenic plants, transgenic plants accumulating it have not yet been obtained (Matoba et al. 2001; Takaiwa 2005). This is due to the rapid clearance in the plant cell after synthesis. Here, our findings indicate that the utilization of A1aB1b as carrier is one of the effective means in producing high amounts of lactostatin in plant. This new transgenic rice variety will be able to serve as a potential functional food in mitigating lifestyle diseases, in particular, hypercholesterolemia. However, improvements can still be done to this new variant of transgenic rice. For example, the number and the positions of lactostatin within A1aB1b can yet be fine-tuned to allow a more soluble and much higher accumulation of lactostatin.

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