Starch synthase IIIa and starch branching enzyme IIb-deficient mutant rice line ameliorates pancreatic insulin secretion in rats: screening and evaluating mutant rice lines with antidiabetic functionalities

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Abstract

Diabetes mellitus is a metabolic disease spreading worldwide that has been reported to worsen the development and progression of other diseases (cancer, vascular diseases and dementia). To establish functional rice lines with anti-postprandial hyperglycaemic effects, we developed mutant rice lines, which lack one or two gene(s) related to starch synthesis, and evaluated their effects. Powder of mutant rice lines or other grains was loaded to rats fasted overnight (oral grain powder loading test). Incremental area under time-concentration curves (iAUC) were calculated with monitored blood glucose levels. Rice lines with anti-postprandial hyperglycaemic effects were separated by cluster analysis with calculated iAUC. A double mutant rice #4019 (starch synthase IIIa (*ss3a*)/branching enzyme IIb (*be2b*)), one of the screened mutant rice lines, was fed to Goto-Kakizaki (GK) rats, an animal model for type 2 diabetes, for 5 weeks. Plasma levels of C-peptide, a marker of pancreatic insulin secretion, were measured with ELISA. For *in vitro* study, a rat pancreatic cell line was cultured with a medium containing rat serum which was sampled from rats fed #4019 diet for 2 d. After 24-h of incubation, an insulin secretion test was performed. Through the oral rice powder loading test, seven rice lines were identified as antidiabetic rice lines. The intake of #4019 diet increased plasma C-peptide levels of GK rats. This result was also observed *in vitro*. In rat serum added to cell medium, ornithine was significantly increased by the intake of #4019. In conclusion, the mutant rice #4019 promoted pancreatic insulin secretion via elevation of serum ornithine levels.

Keywords: Mutant rice lines: Antidiabetic rice: Insulin secretion: C-peptide

Like wheat, rice (*Oryza sativa* L.) is a type of grain eaten worldwide[1]. Especially in Southeast Asia and East Asia, rice is a staple food and an important source of dietary carbohydrates. In Japan, a culture of eating cooked rice as-is, represented by GOHAN (cooked rice), ONIGIRI (rice ball) and DONBURI (rice bowl dishes), is well established. Rice is one of the daily essentials in Japanese life style (compared with other nations). Therefore, huge efforts have been invested into cultivar improvements to enhance the flavour and yieldability of Japonica rice (*O. sativa* L. ssp. *Japonica*) cultivars with

methods such as cross-breeding and differential induction by nitrosomethylurea[2]. As a result, over 500 rice cultivars are registered in the Ministry of Agriculture, Forestry, and Fisheries of Japan. These rice cultivars present various characteristics such as a resistance to high temperature, a better harvest and a more gigantic endosperm[3–5]. Although rice consumption is decreasing in Japan, especially in young people, because of the diversification of dietary life associated with Western wheat-based diet such as breads and pasta[6,7], rice is still an important carbohydrate source in Japan. In addition, global demand of rice is considered to rise

because of its beneficial functionalities for health and the cultural importance of Japanese traditional meals[8,9]. However, it was reported that high-frequency consumption of rice could result in higher risk of type 2 diabetes (T2D), a glucose metabolism disorder increasingly prevalent in Asian countries[10,11]. This concern regarding the health impact prevents people from consuming rice. There is a trend towards low-carbohydrate diet to avoid consuming rice[12–14]. However, advantages and disadvantages of rice for health maintenance are still under debate[15].

Besides morphological phenotypes as described above, rice has a quantitative and qualitative diversity of nutrient components (carbohydrate and proteins) in endosperms[16–18]. Differences in nutrient components in rice endosperm are utilised for the treatment of metabolic diseases. For instance, a cultivar of rice, 'LGC-Jun', is used as functional rice for patient with nephropathy, a protein metabolism disorder[19]. Because the rice 'LGC-Jun' lacks easily digestible proteins (glutelin and globulin) in its endosperm, it is less burdensome in diets for patients with renal disease[19]. Like this instance of 'LGC-Jun', various functionalities are being added to rice lines[16,17,20]. The aim of this study was to screen and evaluate a functional rice line for diabetes. We focused postprandial blood glucose levels as a target in this study. The postprandial blood glucose level is an important factor for the development of T2D, even though increase in blood glucose levels just after diet (a postprandial blood glucose spike) was reported as one of risk factors of T2D and other diseases[21,22]. Furthermore, postprandial glucose level is strongly affected by dietary components. For instance, the higher resistant starch (RS) lowers postprandial blood glucose level[23,24]. Our group previously reported that a rice mutant line with starch branching enzyme IIb-deficiency showed high RS contents when it was boiled[25]. However, how differences in dietary grains, especially rice, impact on postprandial blood glucose levels remains unknown. To evaluate the effects of rice grains on postprandial blood glucose levels, we conducted oral administration tests using rats and then screened antidiabetic rice lines based on cluster analysis with the result of oral administration tests. Furthermore, we fed one of the mutant rice lines categorised as 'antidiabetic', #4019 (starch synthase IIIa (*ss3a*)/branching enzyme IIb (*be2b*)), to Goto-Kakizaki (GK) rats, a model rat of T2D[26]. In addition, we investigated the antidiabetic effect of #4019 *in vivo* and *in vitro*.

Methods

Materials

Rice test samples were provided by Professor N. F. (Akita Prefectural University), Professor T. K. (Kyushu University), and from KAMEDA SEIKA CO., LTD. A rice cultivar 'Nipponbare' was used as the control cultivar because its genome is already sequenced[27]. 'Nipponbare', powdered samples were made from either polished rice or brown rice. Powdered samples of the other rice lines were made from their polished rice endosperms. The characteristics of rice samples, especially mutant rice lines, are shown in Table 1. Hard wheat flour, soft wheat flour, barley flour and durum wheat flour were provided by the Torigoe Co. Ltd. Powder of β-maize starch was purchased from KYUDO Co., LTD. Acarbose (BAY g 5421, O-4, 6-dideoxy-4-(((1S, 4R, 5S, 6S)-4, 5, 6-trihydroxy-3-(hydroxymethyl)-2-cyclohexene-1-yl) amino)-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose) was used as a positive control in Oral cereal powder tolerance test and provided as a tablet, Glucobay 100^{TM} (containing 740 mg Acarbose/g tablet) by Bayer Yakuhin, Ltd.

Animals

Male Sprague–Dawley (SD) rats (Kud:SD), 4 weeks old, for oral cereal powder tolerance test were purchased from KYUDO Co., LTD. Male GK rats, 5 weeks old, were purchased from Japan SLC, Inc.. Rats had free access to a commercial non-purified diet (NMF; Oriental Yeast Co.) and deionised water and were maintained in a temperature-controlled room at 22–25°C with a 12-h light cycle (08.00–20.00 hours). All animal experiments were conducted according to the Guidelines for Animal Experiments Kyushu University and the law (no. 105) and notification (no. 6) of the government of Japan. The animal protocol was approved by the review committee of Kyushu University (authorisation no.: A26-098-3).

Oral cereal powder tolerance test

Oral cereal powder tolerance test was performed with SD rats (male, between 5 and 7 weeks old). To obtain stable results and to reduce the number of rats used, we used rats repeatedly

Table 1. Characteristics of test mutant rice lines

Samples	Deficient gene(s)	Characters about starch structure	Apparent amylose (%)	References
e1	*ss3a*	Decrease in long-chain amylopectin content	30·7	[28], [29]
#2012	*ss3a/ss4b*	Change in shapes of starch grains (polygonal to sphere), decrease in long-chain amylopectin than e1	33·3	[30]
#4012	*ss3a/be1*	High-amylose contents	35·0	(Unpublished)
#4015	*ss3a/be2a*	Similar characters as e1	32·7	(Unpublished)
#4017	*ss1^L/be2b*	High resistant starch contents	21·6	[31]
#4019	*ss3a/be2b*	High resistant starch contents	45·1	[29]
#6002	*ss1^L/ss3a*	Decrease in long-chain amylopectin content	33·4	[32]
EM10	*be2b*	High resistant starch contents	28·1	[29], [33]
EM21	*wx*	Amylose free	0	[34]

ss3a, starch synthase IIIa; *ss4b*, starch synthase IVb; *be1*, branching enzyme I; *be2a*, branching enzyme IIa; *be2b*, branching enzyme IIb; *ss1*, starch synthase I (*ss1^L* is *ss1* leaky mutant); *wx*, waxy (also known as *gbss1*, granule-bound starch synthase I).

(once a week, for 3 weeks). For the test with thirty-three samples, a total of fifty-five SD rats (five rats/sample, for three tests/rat) were used in this study. Each cereal powder (2·0 g/kg body weight (b.w.) suspended in 1·5 ml of ultrapure water) was loaded to SD rats fasted 16 h with syringes attached to 4 Fr catheters (syringe; Terumo Corporation and catheter; Atom Medical Company). As a positive control, acarbose (1·43 mg/kg b.w.) as powdered Glucobay 100^{TM} was administered with 2·0 g/kg b.w. of Nipponbare sample. Blood glucose levels were measured with a glucometer (Accu-Chek® Aviva Nano Blood Glucose Meter System; Roche) until 300 min after loading. After tests, incremental areas under the time-concentration curve (iAUC) between 0 and 300 min (iAUC$_{0–300\,min}$), iAUC between 0 and 30 min (iAUC$_{0–30\,min}$, as an index for glucose absorption), and iAUC between 30 and 90 min (iAUC$_{30–90\,min}$, as an index for blood glucose clearance) were calculated with measured blood glucose levels.

Experimental feeding of mutant rice #4019 to Goto-Kakizaki rats

GK rats were fed a non-purified diet and deionised water for a week and then divided into two groups based on body weight, fasting blood glucose level, and food intake. Rats were fed experimental diet (*n* 7) or control diet (*n* 7) during a limited period (17.00–09.00 hours) for 5 weeks. Diet compositions are presented in Table 2. Food and water intakes were measured once a day, every 2 d, respectively. Rats were euthanized by decapitation after 12 h fasting at the end of the experiment. Blood was collected into tubes and kept in ice after standing at room temperature for 30 min. For plasma preparation, 1 ml of blood was mixed with 50 µl of heparin (Mochida Pharmaceutical Co., Ltd) and 20 µl of DPP-4 inhibitor (EMD Millipore) and then kept on ice. Each blood sample was centrifuged at 1500 **g**, 4°C for 15 min to separate serum and plasma, respectively. Organs (the liver, pancreas, white adipose tissues (WAT) and a part of the abdominal muscle) were excised, weighted, and then immediately frozen in liquid N$_2$. Mesenteric, perirenal, retroperitoneal, epididymal and subcutaneous WAT were excised. The samples (serum, plasma and organs) were kept at −30°C until analysis.

Table 2. Diet composition for #4019 feeding test using Goto-Kakizaki rats

Ingredients	(g/kg diet)	
	Control	#4019
Sucrose	99·5	99·5
Casein	200·	200·
Rice flour (Nipponbare)	500·	–
Rice flour (#4019)	–	500·
Lard	90·	90·
Maize oil	10·	10·
Cellulose	50·	50·
Mineral mix (AIN-76)	35·	35·
Vitamin mix (AIN-76)	10·	10·
DL-Methionine	3·	3·
Choline bitartrate	2·	2·
Cholesterol	0·5	0·5

Measurement of biochemical parameters in serum and plasma

Serum levels of TAG, total cholesterol, NEFA and glucose were measured by using commercial enzyme assay kits (Cholesterol C-Test, Triglyceride E-Test, NEFA C-Test, Glucose CII-Test; Wako Pure Chemical Industries). Plasma *β*-hydroxybutyrate levels were determined with EnzyChrom^{TM} Ketone Body Assay Kit (Bioassay Systems). Serum and plasma diabetic parameters were measured with ELISA kits (Rat Insulin ELISA KIT (TMB), Rat C-peptide (U-type) ELISA KIT; Shibayagi; rat Glucagon ELISA kit, rat glucagon-like peptide-1 (GLP-1) ELISA kit, Amylase test; Wako Pure Chemical Industries).

Measurement of hepatic TAG levels

Hepatic lipids were extracted by using the method described by Folch *et al.*[35]. TAG contents in extracted lipids were measured with the modified method using Triglyceride E-Test as described below. In brief, 0·5 ml of lipid extracts were dried with N$_2$ gas and then suspended in 20 µl of 1:1 mixture solution of Triton X-100 and methanol. After evaporation of the methanol with N$_2$ gas in sample solutions, 200 µl of deionised water and 1·5 ml of colouring reagent were added. Solutions were incubated at 37°C for 5 min. Absorbance values at 600 nm were measured with an UV-VIS spectrophotometer (UV-1600; Shimadzu Corporation).

Evaluation of the effect of #4019 on insulin secretion using rat pancreas cells

SD rats (*n* 4/diet) were trained to consume the basal diet twice/d between 10.00 and 11.00 and 16.00 and 17.00 hours for 2 d. After morning feeding on the 3rd day, the rats were anaesthetised with Somnopentyl® (Kyoritsu Seiyaku Corporation) before blood sampling. At 11.30 hours on the 3rd day, blood samples were obtained from the aorta. Serum samples were separated from obtained blood samples by centrifugation (1500 **g**, 4°C, 5 min × 2 times) and inactivated by incubation at 67°C, for 15 min. The rat pancreas BRIN-BG7 cells (ECACC 10020902) were purchased from DS Pharma Biomedical Co., Ltd[36,37] and cultured with RPMI-1640 medium (Thermo Fisher Scientific) containing 5 % (v/v) fetal bovine serum (FBS) and 5 % rat serum as described above. For maintenance, we used RPMI-1640 medium containing 10 % FBS, not containing rat serum. After 24 h, cells were incubated with low glucose Krebs buffer (1·1 mmol/l glucose) for exactly 40 min and then incubated with high-glucose Krebs buffer (16·7 mmol/l glucose) for exactly 20 min[38]. After incubation, Krebs buffer and cells were collected. C-peptide concentrations in condensed collected buffer were measured with an ELISA kit. Total RNA was obtained from collected cells and expression levels of genes related to insulin secretion were measured by using real-time RT-PCR system (Mx3000P; Agilent Technologies Japan, Ltd) with a SYBR Premix EX Taq II kit (Takara Bio Inc.). The mRNA levels were normalised by using the *β*-actin (*Actb*) gene (for hepatic genes) and the cyclophilin A (*Cypa*) gene (for pancreatic genes) as internal standards. Primer sequences for the analysis are shown in Table 3.

Table 3. Primers for real-time RT-PCR

Genes	Forward primer	Reverse primer
Ins1&2	CCCAGGCTTTGTCAAACAGCA	TTCAGACCTTGGCACTGGAG
Ins1	GTCCTCTGGGAGCCCAAG	CCTGGTGGAGGCTCTGT
Ins2	ATCCTCTGGGAGCCCCGC	CTTGGTGGAAGCTCTCT
Pdx1	GGATGAAATCCACCAAAGCTC	TTCCACTTCATGCGACGGT
Gck	AAGGGAACAACATCGTAGGA	CATTGGCGGTCTTCATCGTA
Mafa	TTCAGCAAGGAGGAGGTCCAT	CCGCCAACTTCTCGTATTTC
Gcg	GATCATTCCCAGCTTCCCAG	GCTGAAGGGACCTTTACCAG
Cd36	GAAGCACTGAAGAATCTGAAGAG	TCCAACACCAAGTAAGACCATC
Fasn	ATGCACACAGTGCTCAAAGG	GTATCCTCCACAGGCAGGAA
Chop	GGAAAGTGGCACAGCTTGCT	TCAGGCGCTCGATTTCCT
Active-Xbp1	GAGTCCGCAGCAGGTG	GCGTCAGAATCCATGGGA
Gpx1	AGAAGTGCGAGGGGAATGGTGA	TTCCAGGAAATGTCGTTGCG
Hmox1	CAGAAGGGTCAGGTGTCCAG	GAAGGCCATGTCCTGCTCTA
Sod1	AGAGGCATGTTGGAGACCTGG	CGGCCAATGATGGAATGCT
Cat	CATGAATGGCTATGGCTCACAC	CAACAGGCAAGTTTTTGATGCC
Cypa	ATTCATGTGCCAGGGTGGTG	CTTCTTGCTGGTCTTGCCATTCC
Actb	TCAGGTCATCACTATCGGCA	TCATGGATGCCACAGGATTC

Analysis of water-soluble metabolites in rat serum (non-target metabolome analysis)

Water-soluble metabolites in rat serum were analysed following the protocol described by Tsugawa et al. [39] with some modifications as described below.

Rat serum (50 µl), Mix solvent (MeOH–ultrapure water–CHCl$_3$ = 5:2:2) (250 µl) and 0·5 mg/ml 2-isopropylmalic acid (10 µl) were mixed in a 1·5-ml microtube. The mixture was shaken with a shaking apparatus at 37°C, 75/min for 30 min. After a centrifugation (16 000 **g**, 4°C, 3 min), 220 µl of supernatant was collected to a new microtube and mixed with 200 µl of ultrapure water. After another centrifugation (16 000 **g**, 4°C, 3 min), 250 µl of supernatant was collected to a new microtube as analysis sample. For the preparation of quality control (QC) samples, 50 µl from each analysis sample were mixed and dispensed to new tubes (200 µl/tube). Analysis samples and QC samples were condensed with a centrifugal thickener (CC-105; Tomy Seiko Co., Ltd) at room temperature until the solvent was evaporated.

Condensed samples were mixed with (or dissolved in) 80 µl of 20 mg/ml methoxyamine hydrochloride (in anhydrous pyridine) and sonicated on ice-cold water bath for 20 min with an ultrasonic cleaner (BRANSON® 5210 ultrasonic cleaner, Branson Ultrasonics; Emerson Japan, Ltd). The mixture was well vortexed and shaken with a shaking apparatus at 30°C, 75/min for 90 min. N-methyl-N-trimethylsilyl-trifluoroacetamide (30 µl) was added to the mixture. The mixture was shaken with a shaking apparatus at 37°C, 75/min for 30 min. After centrifugation (16 000 **g**, 25°C, 5 min), 80 µl of supernatant was collected to 100 µl-spitz attached to a 1·5-ml vial and then sealed with a septum and a screw cap.

Derivatised samples were analysed with GC-MS (Shimadzu GCMS-QP2020 coupled with a InertCap 5MS/NP-VS2 column, 30 m × 0·25 mm, 0·25 µm thickness; GL Sciences). The following temperature programme was applied with helium (high-purity 99·9999 %) as a carrier gas at a flow rate of 1·12 ml/min: 80°C for 2 min; from 80 to 330°C at 15°C/min; 330°C for 6 min. The total run time was 25 min (scanned from 3·5 to 24 min). The injector, the interface, and the ion source were kept at 230, 250 and 200°C, respectively. The mass spectrometer was operated in the electron impact mode (70 eV).

The obtained MS data (qgd-format) were converted to the NetCDF format. With a MetAlign software (free software obtained from the web page of Wageningen University and Research; http://www.wur.nl/nl/show/MetAlign.htm), the peak detection and alignment were performed. The results were saved as a CSV format file. Peak identification and prediction were performed with an AIoutput software (free software obtained from the web page of Riken; http://prime.psc.riken.jp/Metabolomics_Software/). The library file used in peak identification was also downloaded from the web page of RIKEN.

Statistical analysis

All results are shown as means with their standard errors. All data (Tables 4–7, Fig. 2 and 3) were analysed with the χ^2 goodness-of-fit test as a normality test with Excel 2010 (Microsoft) and the add-in software Statcel 3. When the data were recognised as 'non-normal distribution', the data were normalised by logarithmic transformation for further statistical analysis. For comparisons with logarithmic transformation, each geometric mean is shown in Tables 4–7 and Fig. 2(d) and (e). Each median and 95 % CI is shown in each legend (Tables 4–7 and Fig. 2). Data of iAUC$_{0–300\,min}$ were analysed through inspection of differences among rice samples using the Dunnett's test with Excel 2010 and Statcel 3 (Table 4 and Fig. 1(a)). Data of iAUC$_{0–300\,min}$ obtained from other grains and purified carbohydrates were considered as reference data (Table 4). Data in the feeding experiment and in vitro experiment were analysed with Student's t test (Tables 5–7, Fig. 2 and 3) with Excel 2010. Differences were considered significant when P was <0·05. Test cereals were categorised by cluster analysis with data of iAUC$_{0–30\,min}$ and iAUC$_{30–90\,min}$. Cluster analysis was performed with free software 'R' for statistical analysis (http://cran.r-project.org/) (Fig. 1b).

Results

Comparison of incremental area under time-concentration curve between 0 and 300 min obtained with the oral cereal powder tolerance test

Table 4 summarises iAUC values obtained from thirty-three samples. Compared with the $iAUC_{0-300\,min}$ of the control rice line (Nipponbare), those of five rice samples (brown rice of Nipponbare, Koshi-ibuki, #4015, #4019 and EM10) were significantly reduced.

Cluster analysis with incremental area under time-concentration curves between 0 and 30 min and 30 and 90 min obtained from the oral cereal powder tolerance test

As a result of cluster analysis, five clusters were formed (Fig. 1(a) and (b)). Based on the position of Nipponbare and characteristics of samples which belonged to each cluster, we defined each cluster as High-absorption cluster, Normal rice cluster, Antidiabetic cluster, Acarbose cluster and Low-dose glucose cluster, respectively. The antidiabetic cluster contained seven rice lines (#4015, #4019, brown rice of Nipponbare, e1, EM10, koshi-ibuki and mizuhonokagayaki), β-maize starch, durum wheat, rye and barley. Sucrose and 1·0 g/kg b.w. of glucose belonged to Low-dose glucose cluster. EM21, one so-called sticky rice, and 4·0 g/kg b.w. of glucose formed High-absorption cluster. Acarbose formed an independent cluster, the acarbose cluster, by itself.

Effects of the #4019 diet on growth parameters

Intake of #4019 diet did not affect body weight, food intake, water intake, and organ weight, except for perirenal WAT weight (Table 5). Perirenal WAT weight in the #4019 group significantly increased compared with that in the control group.

Table 4. List of the incremental area under time-concentration curves (iAUC) obtained from the oral cereal powder tolerance test† (Mean values with their standard errors, geometric means; *n* 5/group)

Samples	$iAUC_{0-300\,min}$ Mean	$iAUC_{0-300\,min}$ Geometric mean	$iAUC_{0-300\,min}$ SEM	$iAUC_{0-30\,min}$ Mean	$iAUC_{30-90\,min}$ Mean
Rice samples (min × mg/ml)					
1. Nipponbare (control rice)	58·1	54·5	10·7	11·2	34·1
2. Brown rice of Nipponbare	18·2	16·1*	4·3	5·9	11·0
3. IR64 (indica rice)	79·7	79·4	3·2	15·3	36·2
4. Hinohikari	44·6	44·2	2·6	10·8	27·3
5. Yumetoiro	49·1	41·3	7·6	17·8	29·1
6. Akitakomachi	42·5	40·6	6·5	14·6	26·2
7. Eminokizuna	57·3	54·8	8·8	13·0	30·8
8. Mizuhonokagayaki	36·3	35·1	4·2	7·0	21·0
9. Koshi-ibuki	21·1	19·3*	4·5	5·5	11·7
10. e1	30·1	29·8	2·1	10·6	19·9
11. #2012	52·4	51·8	3·9	13·0	31·3
12. #4012	43·9	37·9	11·2	10·7	26·0
13. #4015	17·9	16·8*	2·9	3·4	11·5
14. #4017	63·7	62·9	5·1	10·3	25·2
15. #4019	29·0	28·1*	3·8	9·6	18·8
16. #6002	57·9	53·8	11·4	17·3	32·6
17. EM10	27·5	27·3*	1·9	7·1	15·5
18. EM21	64·7	63·1	6·9	19·8	38·7
19. LGC1	55·0	54·7	3·1	10·9	29·7
20. LGC-Jun	46·8	45·7	4·9	13·5	25·8
Other grains					
21. Rye	24·6		7·2	6·6	15·7
22. Barley	31·9		6·9	7·9	19·6
23. Durum wheat	34·2		5·1	8·4	17·7
24. Hard wheat	38·5		0·2	9·6	23·6
25. Soft wheat	81·9		9·9	7·7	33·3
26. β-Maize starch	23·8		7·9	6·9	16·0
Purified carbohydrates					
27. Maltose	41·2		8·3	12·0	21·5
28. Suclose	28·2		3·8	16·5	9·7
29. Lactose	62·9		12·4	10·0	31·8
30. Glucose 1g/kg b.w.	36·5		2·5	20·4	14·0
31. Glucose 2g/kg b.w.	36·9		4·2	11·2	22·9
32. Glucose 4g/kg b.w.	64·3		9·5	24·5	36·8
Antidiabetic drag (a glucose absorption inhibitor)					
33. Acarbose + Nipponbare	5·0		1·6	2·7	2·3

b.w., Body weight.
* Compared Nipponbare (control rice cultivar) with Dunnett's test (*P* < 0·05).
† Medians of rice samples are as follows: Nipponbare (control rice), 49·7 min × mg/ml (95% CI 28·8, 70·7); brown rice of Nipponbare, 18·2 min × mg/ml (95% CI 9·7, 26·6); IR64 (indica rice), 78·9 min × mg/ml (95% CI 72·6, 85·2); Hinohikari, 43·7 min × mg/ml (95% CI 38·5, 48·8); Yumetoiro, 48·3 min × mg/ml (95% CI 38·0, 58·6); Akitakomachi, 43·5 min × mg/ml (95% CI 30·8, 56·2); Eminokizuna, 51·2 min × mg/ml (95% CI 34·1, 68·4); Mizuhonokagayaki, 41·4 min × mg/ml (95% CI 33·1, 49·6); Koshi-ibuki, 16·1 min × mg/ml (95% CI 7·3, 25·0); e1, 28·8 min × mg/ml (95% CI 24·7, 32·9); #2012, 53·2 min × mg/ml (95% CI 45·4, 60·9); #4012, 33·4 min × mg/ml (95% CI 11·4, 55·3); #4015, 19·7 min × mg/ml (95% CI 14·1, 25·4); #4017, 62·3 min × mg/ml (95% CI 52·3, 72·2); #4019, 25·8 min × mg/ml (95% CI 18·3, 33·3); #6002, 54·2 min × mg/ml (95% CI 32·0, 76·5); EM10, 29·1 min × mg/ml (95% CI 25·4, 32·8); EM21, 63·7 min × mg/ml (95% CI 50·2, 77·2); LGC1, 51·9 min × mg/ml (95% CI 45·9, 57·9); LGC-Jun, 44·6 min × mg/ml (95% CI 35·0, 54·3).
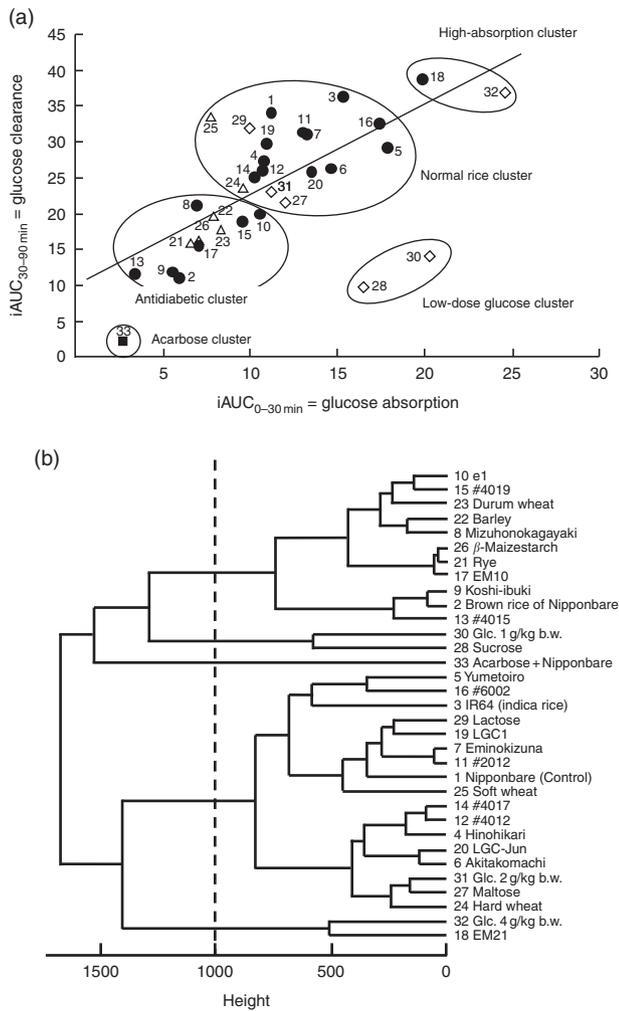
Fig. 1. Cluster analysis dividing samples into five groups. (a) Five clusters on incremental area under time-concentration curve (iAUC) plot. On the iAUC plot, each point means each average of samples (n 5/group) on oral cereal powder tolerance test. Circles on the plot represent clusters as a result of cluster analysis. The numbers on (a) and (b) are obverse. On the plot, different types of symbols represent the difference in sample types (●; rice samples, △; grain samples other than rice, ◇; purified carbohydrate samples, ■; control rice with acarbose). (b) Dendrogram representing the cluster analysis. b.w., body weight.

Effects of the #4019 diet on metabolic parameters

Under fasting conditions, there was no significant difference in serum levels of total cholesterol, TAG, plasma levels of β-hydroxybutyrate, glucose, insulin, GLP-1 and glucagon (Table 6). Liver TAG levels and abdominal muscular glycogen contents did not differ between the groups (Table 6). On the other hand, serum NEFA, plasma C-peptide levels and hepatic glycogen contents were significantly increased in the #4019 diet-fed rats (Table 6).

Effects of the #4019 diet on mRNA levels in the liver and pancreas of Goto-Kakizaki rats

There was no significant difference in mRNA levels of *Fasn*, *Chop*, active-*Xbp1*, *Gpx1*, *Sod1* and *Cat* in the liver of GK rats

fed #4019 diet (Table 7). In addition, pancreatic mRNA levels of *Gcg*, *Cd36*, *Chop*, active-*Xbp1*, *Gpx1*, *Sod1* and *Cat* did not differ between the groups (Table 7). On the other hand, in the pancreas of GK rats fed #4019 diet, the expression of insulin genes (*Ins1* and *Ins2*) was higher than that in rats fed control diet (Table 7). Expression levels of the transcriptional factor, *Pdx1* and *Gck*, a *Pdx1* target, also increased in the #4019 group (Table 7). In addition, #4019 diet significantly increased the expression levels of *Hmox1*, one reactive gene for oxidative stress, when compared with the control diet (Table 6).

Analysis of rat pancreatic cell line cultured in medium containing rat serum

C-peptide concentration in the test buffer tended to increase (P = 0·087) after addition of serum from rats fed #4019 diet (Fig. 2). *Ins* and *Gck* mRNA levels tended to increase (P = 0·073, 0·057, respectively) in BRIN-BG7 cells cultured in #4019 medium. As for GK rats, *Pdx1* expression levels in the cells were significantly increased by addition of #4019 serum (Fig. 2). Unlike the feeding experiment with GK rats, *Hmox1* mRNA levels were not affected by #4019 serum (Fig. 2).

Metabolome analysis of serum used for the *in vitro* study

Relative concentrations of ornithine and glycylglycine were significantly increased in #4019 serum compared with those in control serum (Fig. 3).

Discussion

Recent studies revealed the excessive consumption of carbohydrates, especially rice, is one of risk factors for T2D. However, the simple reduction of rice consumption is difficult because living on rice is not only one of meal types but also one of components of Japanese food culture. The aim of this study is the establishment of an antidiabetic rice cultivar lowering postprandial blood glucose level and the investigation of its mechanism. In this study, we directly compared rice powders of twenty lines, six grain powders and six purified carbohydrates containing three doses of glucose to determine their effects on postprandial blood glucose levels in rats and identified seven highly effective rice lines by cluster analysis. Among rice lines belonging to the antidiabetic cluster, we fed a mutant rice line #4019, which lacks enzymes involved in starch synthesis, to GK rats to evaluate the effects of long-term intake of #4019 on diabetes. Feeding experiment revealed that intake of #4019 promoted insulin secretion from the pancreas of GK rats via increasing pancreatic mRNA expression of *Pdx1*, a transcriptional factor for insulin genes (Tables 6 and 7). This phenomenon was also observed in the *in vitro* experiment with rat pancreatic cells (Fig. 2).

We screened seven rice lines, including four mutants screened as rice lines with small or moderate postprandial blood glucose levels (Fig. 1). Rice samples administered in this study were popular rice cultivars (commercially available in Japan) or mutant rice lines which lack genes involved in starch synthesis. Besides, Koshihikari, one of the most famous

Table 5. Growth parameters of Goto-Kakizaki rats fed experimental diet for 5 weeks†
(Mean values with their standard errors, geometric means; *n* 7/group)

	Control			#4019		
	Mean	Geometric mean	SEM	Mean	Geometric mean	SEM
Initial body weight (g)	143		2	143		3
Final body weight (g)	293		4	293		4
Total food intake (g)	565		6	566		8
Food efficiency (%)	0·265		0·005	0·265		0·004
Total water intake (ml)	613		25	637		25
Organ weight (g)						
Liver	10·5	10·5	0·2	10·8	10·8	0·2
Pancreas	0·995	0·989	0·047	1·03	1·026	0·03
Mesenteric WAT	5·02		0·25	5·2		0·28
Perirenal WAT	0·966	0·961	0·051	1·17	1·16*	0·059
Retroperitoneal WAT	8·29		0·52	8·7		0·47
Epididymal WAT	5·05		0·2	5·33		0·28
Subcutaneous WAT	20·8		0·8	22·1		1·2

WAT, white adipose tissue.
* Significant difference at *P* < 0·05 analysed with Student's *t* test.
† As results of normality test, parameters with non-normal distribution were normalised by logarithmic transformation before statistical analysis with Student's *t* test. Medians of parameters with non-normal distribution are as follows: liver weight (control), 10·4 g (95 % CI 10·1, 10·7); (#4019), 10·7 g (95 % CI 10·2, 11·2); pancreas weight (control), 0·969 g (95 % CI 0·877, 1·061); (#4019), 1·064 g (95 % CI 0·997, 1·131); perirenal WAT (control), 0·990 g (95 % CI 0·883, 1·097); (#4019), 1·110 g (95 % CI 0·995, 1·225).

Table 6. Biochemical parameters of Goto-Kakizaki rats fed #4019†
(Mean values with their standard errors, geometric means; *n* 7/group)

	Control			#4019		
	Mean	Geometric mean	SEM	Mean	Geometric mean	SEM
Serum						
Total cholesterol (mg/ml)	1·66		0·09	1·59		0·08
TAG (mg/ml)	1·16	1·13	0·10	1·50	1·44	0·17
NEFA (mEq/l)	0·613		0·027	0·787*		0·039*
Amylase activity (U/ml)	63518		390	63363		474
Plasma						
β-Hydroxybutyrate (mM)	0·776	0·765	0·053	0·707	0·684	0·076
Glucose (mg/ml)	1·87		0·19	1·78		0·09
Insulin (ng/ml)	4·89	4·77	0·48	4·84	4·75	0·42
C-peptide (pg/ml)	489		52	880*		120*
Glucagon-like peptide-1 (ng/ml)	12	9·42	3·4	8·45	7·95	0·95
Glucagon (pg/ml)	251	219	52	156	153	10
Liver TAG (mg/g tissue)	15·8		0·4	15·3		0·5
Glycogen (mg/g tissue)						
Liver	16·6	16·4	0·9	27·4*	26·8	2·5*
Abdominal muscle	0·462		0·049	0·43		0·035

* Significant difference at *P* < 0·05 analysed with Student's *t* test.
† As results of normality test, parameters with non-normal distribution were normalised by logarithmic transformation before statistical analysis with Student's *t* test. Medians of parameters with non-normal distribution are as follows: serum TAG (control), 1·32 mg/ml (95 % CI 1·04, 1·43); (#4019), 1·37 mg/ml (95 % CI 1·03, 1·70); plasma β-hydroxybutyrate (control), 0·830 mM (95 % CI 0·726, 0·934); (#4019), 0·616 mM (95 % CI 0·466, 0·765); plasma insulin (control), 4·66 ng/ml (95 % CI 3·72, 5·60); (#4019), 4·69 ng/ml (95 % CI 3·88, 5·51); plasma glucagon-like peptide-1 (control), 9·23 ng/ml (95 % CI 1·53, 16·92); (#4019), 8·72 ng/ml (95 % CI 6·58, 10·87); plasma glucagon (control), 173 pg/ml (95 % CI 55, 291); (#4019), 161 pg/ml (95 % CI 139, 184); liver glycogen (control), 17·6 mg/g tissue (95 % CI 15·9, 19·3); (#4019), 28·0 mg/g tissue (95 % CI 23·0, 32·9).

cultivars in Japan, was not administered because its genetic background is not homogenous. Among highly effective rice lines other than EM10, *ss3a* was commonly deficient. We previously reported that *ss3a* deficiency leads to high-amylose content in rice endosperm[28]. A starch structure in #4015 endosperm (A mutant rice with the strongest lowering effect; *ss3a*/branching enzyme IIa (*be2a*), sample 13) was predicted to be similar because the effect of *be2a* deficiency to rice endosperm is weak[40]. In addition, EM10 (*be2b*, sample 17)

has been reported to have high-amylose content[29] and high-RS content[41]. The antidiabetic functionality of these mutant lines was considered to be glucoamylase-resistance from high-amylose contents. Interestingly, the antidiabetic effect of #2012 (*ss3a*/starch synthase IVb, sample 11) and #4012 (*ss3a*/branching enzyme I, sample 12), which also have a deficiency in *SSIIIa*, was weak (Fig. 1(a)). Taken together, the effect of *SSIIIa* deficiency changed depending on the co-deficient gene. We hypothesised that *SSIIIa* deficiency

Table 7. Gene expression levels in tissues of Goto-Kakizaki rats fed #4019†
(Mean values with their standard errors, geometric means; n 7/group)

	Control			#4019		
	Mean	Geometric mean	SEM	Mean	Geometric mean	SEM
Gene expressions in the liver (arbitrary units)						
Fasn	1·00	0·84	0·27	1·66	1·06	0·65
Chop	1·00		0·13	0·92		0·29
active-Xbp1	1·00	0·83	0·29	1·11	1·00	0·20
Gpx1	1·00		0·17	0·84		0·11
Sod1	1·00		0·13	0·98		0·15
Cat	1·00		0·08	1·01		0·11
Gene expressions in the pancreas (arbitrary units)						
Ins1	1·00		0·12	1·62*		0·24
Ins2	1·00		0·12	1·74*		0·25
Pdx1	1·00		0·25	2·31*		0·50
Mafa		ND			ND	
Hmox1	1·00	0·96	0·13	1·66*	2·08	0·14
Gcg	1·00		0·17	1·62		0·31
Cd36	1·00		0·16	1·1		0·08
Chop	1·00		0·07	1·48		0·21
active-Xbp1	1·00		0·08	1·05		0·14
Gpx1	1·00	0·97	0·11	1·12	1·06	0·18
Sod1	1·00		0·1	1·13		0·13
Cat	1·00	0·94	0·15	1·01	0·99	0·10

ND, not detected. * Significant difference at $P < 0.05$ analysed with Student's t test.
† As results of normality test, parameters with non-normal distribution were normalised by logarithmic transformation before statistical analysis with Student's t test. Medians of parameters with non-normal distribution are as follows: liver Fasn (control), 0·662 (95 % CI 0·133, 1·191); (#4019), 1·471 (95 % CI 0·196, 2·745); liver active Xbp1 (control), 0·666 (95 % CI 0·088, 1·244); (#4019), 0·992 (95 % CI 0·605, 1·378); pancreas Hmox1 (control), 1·064 (95 % CI 0·855, 1·273); (#4019), 1·671 (95 % CI −0·124, 3·467); pancreas Gpx1 (control), 0·911 (95 % CI 0·689, 1·134); (#4019), 0·978 (95 % CI 0·624, 1·332); pancreas Cat (control), 0·869 (95 % CI 0·570, 1·169); (#4019), 0·967 (95 % CI 0·770, 1·165).

in rice is related to insulin secretion in animals fed with the rice, from a comparison of the results obtained with e1 (*ss3a*, sample 10) and #4012 (high iAUC$_{30–90\,min}$ despite similar iAUC$_{0–30\,min}$).

We focused on the double mutant rice #4019 (*ss3a/be2b*, sample 15) among the seven screened antidiabetic rice lines and fed #4019 to GK rats, a model rat for T2D, for 5 weeks. Although #4019 feeding did not affect serum glucose levels and serum hormone levels (insulin, glucagon, and GLP-1), plasma C-peptide levels in the #4019-fed group were significantly higher than those in the control (Nipponbare-fed) group. C-peptide is the co-product of insulin maturation, and has a longer half-life than insulin[42]. Therefore, C-peptide in blood samples is considered a marker for insulin secretion. Higher plasma C-peptide levels suggested that ingestion of #4019 activated insulin secretion from pancreatic islet β-cells in the #4019 group although serum insulin levels were not changed probably due to 12-h fasting before being killed. Promotion of insulin secretion by #4019 was also observed *in vitro* (Fig. 2). Both *in vivo* and *in vitro* studies indicated that the expression of insulin genes (*Ins1* and *Ins2*) and their positive transcriptional factor, *Pdx1*, was induced by #4019 ingestion. These results suggested that ingestion of #4019 promoted insulin secretion from the pancreas via promotion of *Pdx1* expression in pancreatic cells.

The *in vitro* experiment suggested two points. One is that a mutant rice #4019 promotes *Ins1* and *Ins2* expression via *Pdx1*. The other is that a responsible substance is a substance that appears in blood serum after ingestion of #4019. Ferulic acid and γ-oryzanol are well-known functional components in

rice[43,44]. However, these substances are predominantly contained in rice bran not in rice endosperm because of their hydrophobicity[45]. We hypothesised that the responsible substance was a water-soluble metabolite in rice endosperm and performed a non-target metabolome analysis for water-soluble metabolites in rat serum used for the *in vitro* study. The metabolome analysis revealed that glycylglycine and ornithine concentrations in serum were significantly increased by intake of #4019 (Fig. 3). In the body, ornithine is synthesised from arginine by arginase in urea cycle. An increased arginine contents in #4019 and activation of arginase by intake of #4019 are suspected as one of possible mechanisms for high ornithine levels. In the serum of #4019-fed rats, allantoin levels, a metabolite of uric acid[46], were tended to increase (about 1·5 times higher, $P = 0.057$, data not shown). This increase suggested the promotion of urea cycle and arginine conversion to ornithine. To clarify the mechanism, further studies are needed. Although ornithine is known to promote insulin-like growth factor[47], Leiss *et al.*[48] recently reported that ornithine can also stimulate insulin secretion via $G\alpha_{i2}$, belonging to the G-protein family. Taken together, we consider that ornithine increased by #4019 ingestion contribute to the promotion of insulin secretion from the pancreas. Although the serum concentrations of metabolites were not measurable by non-target metabolomics, it is possible to estimate the increase in serum ornithine concentration based on the literature. Vadivel *et al.*[49] reported that ornithine concentration in rat sera was about 100 μmol/l. Our metabolomic analysis showed that the signal intensity of ornithine in #4019-fed rat was 1·4-time stronger than that in control rats (Fig. 3). From this result, serum ornithine concentration was
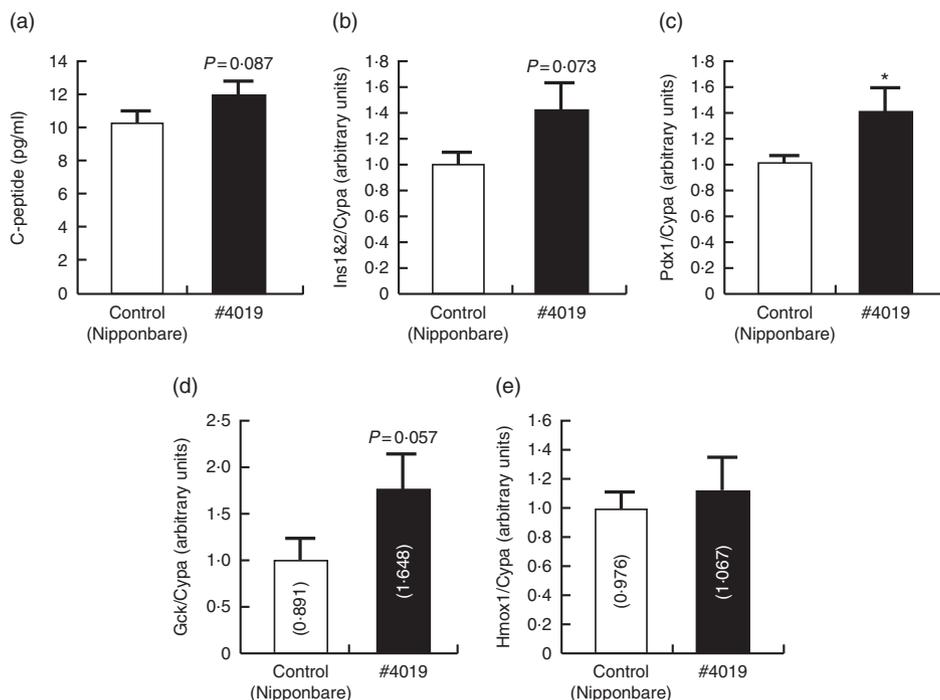
Fig. 2. Serum from Sprague–Dawley rats being fed #4019 (*ss3a/be2b*) diet promoted insulin secretion from rat pancreatic cell by promoting *Pdx1* expression. (a) C-peptide levels in high-glucose test buffer, (b–e) cellular mRNA levels of genes related to the regulation of insulin secretion. Values are means (*n* 6/group), with their standard errors represented by vertical bars. As results of normality test, parameters with non-normal distribution were normalised by logarithmic transformation before statistical analysis with Student's *t* test. The values in bars in (d) and (e) are geometric means. Medians of parameters with non-normal distribution are as follows: *Gck* (control), 0·894 (95 % CI 0·456, 1·332); (#4019), 1·452 (95 % CI 0·733, 2·172); *Hmox1* (control), 0·853 (95 % CI 0·650, 1·055); (#4019), 0·966 (95 % CI 0·560, 1·372). * Significant difference at $P < 0.05$ analysed with Student's *t* test.
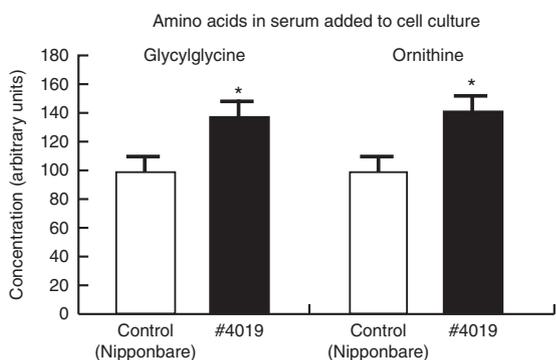

Fig. 3. The ingestion of #4019 (*ss3a/be2b*) diet increased glycylglycine and ornithine concentration in the rat serum. Values are means (*n* 4/group), with their standard errors represented by vertical bars. The values of control groups were set to 100. * Significant difference at $P < 0.05$ analysed with Student's *t* test.

estimated at approximately 140 µmol/l (a 40 µmol/l increase). An i.p. administration of 10 mM L-ornithine at 100 µl (equal to 132 µg L-ornithine) to fasted rats induced a 0·1 µg/l increase of serum insulin concentration in under a minute although plasma L-ornithine concentration was not shown in the report[48]. The 40 µmol/l increase in serum L-ornithine concentration in the *in vitro* study was equal to an increase of 10·6 µg and considered reasonable for an about 4 ng/l increase of C-peptide secretion. However, whether *Pdx1* expression is promoted by activation of the G-protein signal system remains unknown. As described above, *ss3a* and *be2b*, deficient genes in #4019,

codes starch-synthesising enzymes[29]. Redestig *et al.*[50] investigated the relationships between rice quality traits and various metabolites in kernels (as brown rice) by a metabolomic analysis with seven rice samples, including e1 and #4019. They reported that various metabolites in rice (stearic acid, phosphoric acid, lysine and ornithine) were involved in the determination of amylose/amylopectin ratio in brown rice[50]. Although there were some differences between polished white rice and brown rice, it is predicted that starch structure and metabolites in endosperm are related to each other. Taken together, mutations in *ss3a* and *be2b* changed endosperm contents of metabolites via alteration of the starch structure. However, there are still possibilities that another substance in rice endosperm contributed to induce pancreatic expression of insulin-related genes. Further study is necessary to identify the responsible substance in #4019 for promotion of insulin expression.

In oral cereal powder tolerance test for screening, samples of other grains (three types of wheat, barley, rye and *β*-maize starch) were tested in the same system. Although the differences between the cultivars were ignored, increases in blood glucose levels by these samples, other than soft wheat, were relatively small compared with those induced by popular rice cultivars (Nipponbare, Akitakomachi and Hinohikari) (Fig. 1(a), Table 4). Among six loaded samples of non-rice grains, four samples (durum wheat, barley, rye and *β*-maize starch) belonged to the antidiabetic cluster (Fig. 1(a), Table 4). Although hard and soft wheat belonged to normal cluster, their iAUC$_{0-30\,min}$,

recognised as an index for glucose absorption, were similar to those of durum wheat. These results indicated that consumption of popular rice cultivars resulted in higher postprandial blood glucose levels than other grains. According to *Standard Tables of Food Composition in Japan* (7th ed.), carbohydrate contents are similar among grains (rice: 74·5 g/100 g edible part, lay: 75·8 g/100 g, barley: 77·1 g/100 g and maize starch: 86·3 g/100 g)[51]. In this screening test, the same amount of grains was loaded to rats. Differences in postprandial blood glucose levels, especially $iAUC_{0–30 min}$, were determined mainly by differences in digestion efficiencies of loaded samples. Higher $iAUC_{0–30 min}$, reflecting greater glucose absorption, by rice samples meant rice-starch was easier to digest than starch in other grains. This tendency of rice-starch results from cultivar improvements to pursue better taste, which have been operated through the ages in Japan. To improve taste, the ratio of amylopectin in rice-starch was increased by traditional cultivar improvements to achieve easier digestion by salivary amylase[52]. This is supported by the result of EM21. Almost all starches in endosperm of EM21, one of the rice lines called sticky rice, are amylopectin[33]. Taken together, development of an antidiabetic rice line may be equal to a reversal process of development of good taste cultivars traditionally developed in Japan.

In oral cereal powder tolerance test, iAUC of 2 g/kg b.w. EM21 administration and 4 g/kg b.w. glucose were in almost the same range (Table 4, Fig. 1(a)). Not applied only to EM10, rice samples belonging to the normal cluster resulted in higher iAUC than 2·0 g/kg b.w glucose (Table 4, Fig. 1(a)). These results implied an interesting possibility about rice. In this study, we administered raw grain samples because rats present high amylase activities sufficient to digest raw rice. Raw rice endosperm contains about 80 wt% carbohydrate[51]; thus, 2·0 g of rice contains 1·6 g of glucose at maximum. Thus, larger-than-expected increases by rice administration could not be explained only as the result of its easily digestible starch-structure, and implied that rice contained substance(s) which inhibited insulin secretion or insulin sensitivity. Further study is necessary to confirm this hypothesis and identify the responsible substances in rice.

In this study, we used iAUC plot, which enabled us to simultaneously estimate both of increases and clearances of blood glucose after sample loading. Separation of iAUC at 30 min, not at peak times of postprandial blood glucose levels, makes separation effective. Plots of samples which result in rapid peaks and quick clearances of blood glucose such as 1 g/kg b.w. sucrose located in the lower right area (Fig. 1(a)). On the other hand, plots of samples which result in moderate increases and clearances of blood glucose like lactose located higher in the left area (Fig. 1(a)). We believe that iAUC plot is quite useful for the screening of antidiabetic foods.

In conclusion, in this study, we separated seven highly effective rice samples from twenty rice samples by oral cereal powder tolerance test with other grains and six purified carbohydrates. One of the rice samples belonging to the antidiabetic cluster, #4019, was fed to GK rats. Feeding experiments revealed that intake of #4019 promoted insulin secretion from the pancreas via activation of *Pdx1*. Intake of #4019 instead of other popular rice cultivars is expected to be a useful approach for the prevention of T2D. In addition, our methodology in this study will be available for the establishment of other functional cultivars.

References

1. Department of Agriculture & Economic Research Service (2017) Data sets. https://www.fas.usda.gov/data/grain-world-markets-and-trade (accessed March 2017).
2. Satoh H & Omura T (1979) Induction of mutation by the treatment of fertilized egg cell with N-methyl-N-nitrosourea in rice. *J Fac Agr Kyushu Univ* **24**, 165–174.
3. Wada T, Tsubone M, Inoue T, et al. (2010) 'Genkitsukushi', a new rice [*Oryza sativa*] cultivar with tolerance to high temperature during maturing period and high eating quality. *Bull Fukuoka Agric Res Cent* **29**, 1–9.
4. Okamoto K, Nishimiya T, Hirayama M, et al. (2015) A new rice variety 'Fukumaru'. *Bull Ibaraki Plant Biotech Inst* **15**, 33–40.
5. Maeda H, Nemoto H, Iida S, et al. (2001) A new rice variety with giant embryos, 'Haiminori'. *Breed Sci* **51**, 211–213.
6. Melby MK & Takeda W (2014) Lifestyle constraints, not inadequate nutrition education, cause gap between breakfast ideals and realities among Japanese in Tokyo. *Appetite* **72**, 37–49.
7. Adachi M (2008) Theories of nutrition education and promotion in Japan: enactment of the 'Food Education Basic Law'. *Asia Pac J Clin Nutr* **17**, Suppl. 1, 180–184.
8. Hamajima H, Matsunaga H, Fujikawa A, et al. (2016) Japanese traditional dietary fungus koji *Aspergillus oryzae* functions as a prebiotic for Blautia coccoides through glycosylceramide: Japanese dietary fungus koji is a new prebiotic. *Springerplus* **5**, 1321.
9. Funatsu S, Kondoh T, Kawase T, et al. (2015) Long-term consumption of dried bonito dashi (a traditional Japanese fish stock) reduces anxiety and modifies central amino acid levels in rats. *Nutr Neurosci* **18**, 256–264.
10. Golozar A, Khalili D, Etemadi A, et al. (2017) White rice intake and incidence of type-2 diabetes: analysis of two prospective cohort studies from Iran. *BMC Public Health* **17**, 133.
11. Saneei P, Larijani B & Esmaillzadeh A (2017) Rice consumption, incidence of chronic diseases and risk of mortality: meta-analysis of cohort studies. *Public Health Nutr* **20**, 233–244.
12. Foster GD, Wyatt HR, Hill JO, et al. (2003) A randomized trial of a low-carbohydrate diet for obesity. *N Engl J Med* **348**, 2082–2090.
13. Czyżewska-Majchrzak Ł, Grzelak T, Kramkowska M, et al. (2014) The use of low-carbohydrate diet in type 2 diabetes - benefits and risks. *Ann Agric Environ Med* **21**, 320–326.
14. Hickey JT, Hickey L, Yancy WS, et al. (2003) Clinical use of a carbohydrate-restricted diet to treat the dyslipidemia of the metabolic syndrome. *Metab Syndr Relat Disord* **1**, 227–232.

15. Eshak ES, Iso H, Date C, *et al.* (2011) Rice intake is associated with reduced risk of mortality from cardiovascular disease in Japanese men but not women. *J Nutr* **141**, 595–602.

16. Mizuno H, Fukumori T, Liu K, *et al.* (2012) The influence of heating and humidifying by humidified air on GABA generation and quality in paddy. *J Jpn Soc Agric Mach* **74**, 226–233 (in Japanese).

17. Mizuno H, Fukumori T, Liu K, *et al.* (2012) Development of GABA enriching equipment for brown rice by heating and humidifying of moist air. *J Jpn Soc Agric Mach* **74**, 234–243 (in Japanese).

18. Jeng TL, Ho PT, Shih YJ, *et al.* (2011) Comparisons of protein, lipid, phenolics, γ-oryzanol, vitamin E, and mineral contents in bran layer of sodium azide-induced red rice mutants. *J Sci Food Agric* **91**, 1459–1465.

19. Nishimura M, Kusaba M, Miyahara K, *et al.* (2005) New rice varieties with low levels of easy-to-digest protein, 'LGC-Katsu' and 'LGC-Jun'. *Breed Sci* **55**, 103–105.

20. Ogo Y, Wakasa Y, Hirano K, *et al.* (2014) Generation of transgenic rice with reduced content of major and novel high molecular weight allergens. *Rice (N Y)* **7**, 19.

21. Ceriello A, Esposito K, Piconi L, *et al.* (2008) Glucose 'peak' and glucose 'spike': Impact on endothelial function and oxidative stress. *Diabetes Res Clin Pract* **82**, 262–267.

22. Bonora E & Muggeo M (2001) Postprandial blood glucose as a risk factor for cardiovascular disease in Type II diabetes: the epidemiological evidence. *Diabetologia* **44**, 2107–2114.

23. Brites CM, Trigo MJ, Carrapiço B, *et al.* (2011) Maize and resistant starch enriched breads reduce postprandial glycemic responses in rats. *Nutr Res* **31**, 302–308.

24. Klosterbuer AS, Thomas W & Slavin JL (2012) Resistant starch and pullulan reduce postprandial glucose, insulin, and GLP-1, but have no effect on satiety in healthy humans. *J Agric Food Chem* **60**, 11928–11934.

25. Tsuiki K, Fujisawa H, Itoh A, *et al.* (2016) Alterations of starch structure lead to increased resistant starch of steamed rice: identification of high resistant starch rice lines. *J Cereal Sci* **68**, 88–92.

26. Goto Y, Kakizaki M & Masaki N (1976) Production of spontaneous diabetic rats by repetition of selective breeding. *Tohoku J Exp Med* **119**, 85–90.

27. International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. *Nature* **436**, 793–800.

28. Fujita N, Yoshida M, Kondo T, *et al.* (2007) Characterization of SSIIIa-deficient mutants of rice: The function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm. *Plant Physiol* **144**, 2009–2023.

29. Asai H, Abe N, Matsushima R, *et al.* (2014) Deficiencies in both starch synthase IIIa and branching enzyme IIb lead to a significant increase in amylose in SSIIa-inactive japonica rice seeds. *J Exp Bot* **65**, 5497–5507.

30. Toyosawa Y, Kawagoe Y, Matsushima R, *et al.* (2016) Deficiency of starch synthase IIIa and IVb alters starch granule morphology from polyhedral to spherical in rice endosperm. *Plant Physiol* **170**, 1255–1270.

31. Abe N, Asai H, Yago H, *et al.* (2014) Relationships between starch synthase I and branching enzyme isozymes determined using double mutant rice lines. *BMC Plant Biol* **14**, 80.

32. Hayashi M, Kodama M, Nakamura Y, *et al.* (2015) Thermal and pasting properties, morphology of starch granules, and crystallinity of endosperm starch in the rice SSI and SSIIIa double-mutant. *J Appl Glycosci* **62**, 81–86.

33. Nishi A, Nakamura Y, Tanaka N, *et al.* (2001) Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. *Plant Physiol* **127**, 459–472.

34. Ando R, Kageshima F, Takaguchi H, *et al.* (2013) Food application tests of starches in waxy mutant rice lines. *Jpn Soc Appl Glycosci* **3**, 213–221 (in Japanese).

35. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497–509.

36. Clarke J, McClenaghan NH, Barnett CR, *et al.* (1995) BRIN-BG7, a novel glucose-responsive insulin-secreting cell-line produced by electrofusion. *Diabetologia* **38**, A108–A108.

37. McClenaghan NH, Barnett CR, O'Harte FP, *et al.* (1996) Characteristics of BRIN-BG5 and BRIN-BG7, two novel glucose-responsive insulin-secreting cell lines produced by electrofusion. *J Endocrinol* **148**, 409–417.

38. Moran BM, Abdel-Wahab YH, Flatt PR, *et al.* (2014) Evaluation of the insulin-releasing and glucose-lowering effects of GPR120 activation in pancreatic β-cells. *Diabetes Obes Metab* **16**, 1128–1139.

39. Tsugawa H, Bamba T, Shinohara M, *et al.* (2011) Practical non-targeted gas chromatography/mass spectrometry-based metabolomics platform for metabolic phenotype analysis. *J Biosci Bioeng* **112**, 292–298.

40. Nakamura Y (2002) Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. *Plant Cell Physiol* **43**, 718–725.

41. Nakamura S, Satoh H & Ohtsubo K (2010) Palatable and bio-functional wheat/rice products developed from pre-germinated brown rice of super-hard cultivar EM10. *Biosci Biotechnol Biochem* **74**, 1164–1172.

42. Matthews DR, Rudenski AS, Burnett MA, *et al.* (1985) The half-life of endogenous insulin and C-peptide in man assessed by somatostatin suppression. *Clin Endocrinol (Oxf)* **23**, 71–79.

43. Jung EH, Kim SR, Hwang IK, *et al.* (2007) Hypoglycemic effects of a phenolic acid fraction of rice bran and ferulic acid in C57BL/KsJ-db/db mice. *J Agric Food Chem* **55**, 9800–9804.

44. Yu S, Nehus ZT, Badger TM, *et al.* (2007) Quantification of vitamin E and gamma-oryzanol components in rice germ and bran. *J Agric Food Chem* **55**, 7308–7313.

45. Zaima N, Goto-Inoue N, Hayasaka T, *et al.* (2010) Application of imaging mass spectrometry for the analysis of *Oryza sativa* rice. *Rapid Commun Mass Spectrom* **24**, 2723–2729.

46. Wu XW, Lee CC, Muzny DM, *et al.* (1989) Urate oxidase: primary structure and evolutionary implications. *Proc Natl Acad Sci U S A* **86**, 9412–9416.

47. Zajac A, Poprzecki S, Zebrowska A, *et al.* (2010) Arginine and ornithine supplementation increases growth hormone and insulin-like growth factor-1 serum levels after heavy-resistance exercise in strength-trained athletes. *J Strength Cond Res* **24**, 1082–1090.

48. Leiss V, Flockerzie K, Novakovic A, *et al.* (2014) Insulin secretion stimulated by ʟ-arginine and its metabolite ʟ-ornithine depends on Gα(i2). *Am J Physiol Endocrinol Metab* **307**, E800–E812.

49. Vadivel A, Aschner JL, Rey-Parra GJ, *et al.* (2010) ʟ-Citrulline attenuates arrested alveolar growth and pulmonary hypertension in oxygen-induced lung injury in newborn rats. *Pediatr Res* **68**, 519–525.

50. Redestig H, Kusano M, Ebana K, *et al.* (2011) Exploring molecular backgrounds of quality traits in rice by predictive models based on high-coverage metabolomics. *BMC Syst Biol* **5**, 176.

51. Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan (2015) *Standard Tables of Food Composition in Japan 2015*, 7th ed. http://www.mext.go.jp/a_menu/syokuhinseibun/1365297.htm (in Japanese with English translation) (accessed February 2018).

52. Okuno K, Fuwa H & Yano M (1983) A new mutant-gene lowering amylose content in endosperm starch of rice, *Oryza sativa* L. *Jpn J Breed* **33**, 387–394 (in Japanese).