Genetically modified rice seeds accumulating GLP-1 analogue stimulate insulin secretion from a mouse pancreatic beta-cell line

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Abstract Glucagon-like peptide-1 (7-36) amide (GLP-1) is the most potent physiological insulinotropic hormone in humans. We produced large amounts of a GLP-1 analogue, [Ser^{8}, Gln^{26}, Asp^{34}]-GLP-1, which is resistant to trypsin-digestion, as part of a chimeric rice seed storage protein, a 26 kDa globulin, in genetically modified rice seeds. Junction sites between GLP-1 analogue and globulin were replaced by tryptic cleavage sites. The highest level of GLP-1 analogue accumulation was ≈20–50 μg per seed. We found that GLP-1 analogue derived from trypsin-digested genetically modified rice seeds stimulated insulin secretion from a mouse pancreatic beta-cell line, MIN6.

Keywords: Glucagon-like peptide-1; Insulin; Diabetes; Globulin; Genetically modified rice

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a potent blood glucose-lowering hormone that stimulates the secretion of insulin from pancreatic beta-cells [1,2]. Since the half-life of GLP-1 by subcutaneous injections is very short [3,4], novel GLP-1 analogues or a synthetically produced GLP-1 agonist, Exendin-4 with a binding affinity to the pancreatic GLP-1 receptor similar to that of GLP-1 have been developed [5]. Another approach includes the use of dipeptidylpeptidase-IV(DPPIV) inhibitors to augment the endogenous active GLP-1 [6] that was instantly cleaved by DPPIV at Ala^{8}. We describe here a new approach for the exogenous administration of GLP-1 analogue, in which patients with Type 2 diabetes eat genetically modified rice seeds with elevated levels of the GLP-1 analogue.

Medical molecular farming has been an attractive possible option for the production of recombinant biopharmaceuticals in contrast to methods using animal cells, bacteria, and fungi [7–9]. On the other hand, the stable storage of small peptides can be very difficult to achieve in genetically modified plant systems. Vandekerckhove et al. [10] reported an elegant system in which 2S albumin–enkephalin (pentapeptide) fusion protein was produced in the seeds of genetically modified *Arabidopsis* and oilseed. In this study, we constructed a chimeric globulin seed storage protein gene. In this DPPIV- and trypsin-resistant GLP-1 analogue, the DPPIV sensitive site Ala^{8} was changed to Ser^{8} and two tryptic cleavage sites, Lys^{26} and Lys^{34}, were changed to Gln^{26} and to Asp^{34}, respectively. This construct was inserted into the variable region of the rice globulin gene under control of the native globulin promoter. To release the active GLP-1 analogue in the small intestine, tryptic cleavage sites were introduced to both ends of the GLP-1 analogue gene. We generated Genetically modified rice seeds were generated using the MAT-vector system [11,12].

In the present report, rice seeds containing the GLP-1 analogue as part of a chimeric globulin seed storage protein were digested with trypsin and the trypsin-resistant GLP-1 analogue was extracted. The content of the GLP-1 analogue that had been accumulated was measured by radioimmunoassays. In vitro studies of a mouse pancreatic beta-cell line demonstrated that GLP-1 analogue from chimeric globulin stimulated insulin secretion in a mouse pancreatic beta-cell line, MIN6 [13]. These findings suggest that the consumption of genetically modified rice seeds containing elevated levels of the GLP-1 analogue may control blood glucose levels in humans.

2. Materials and methods

2.1. Plasmid construction

Plasmids were constructed using standard recombinant technique [14]. The codon-optimized GLP-1 analogue gene was synthesized by PCR based on the designed sequence using long sense and antisense oligonucleotides with overlaps of 18 nt as primers using high fidelity Taq polymerase. This GLP-1 analogue gene was introduced between amino acid positions 109 and 110 of 26 kDa globulin cDNA by two-overlap extension PCR method, and then fused to 980 bp globulin promoter. The *Eco*RI–*Sse*8387I fragment of *Glb* promoter-globulin (GLP-1 analogue)-Nos terminator was ligated into the *Eco*RI–*Sse*8387I site of pTL7 to produce pGlbGLP. The *Kpn*I fragment of the CaMV35S promoter-*hph*-Nos terminator was ligated into the internal *Kpn*I site of the *R*/RS cassette of pNPI130PUC to produce pNPI130Hm [12]. The *Sse*8387I fragment of pNPI130Hm was cloned into the *Sse*8387I site of pGlbGLP to produce pGlbGLP130Hm.

2.2. Transformation

This pGlbGLP130Hm was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation [15].

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Transformation and regeneration of shoots were carried out according to the modified procedure described in [16]. Mature rice seeds (*Oryza sativa* L.cv. Nipponbare) were sterilized in 75% (v/v) sodium hypochloride for 30 min and washed thoroughly in sterilized water. The sterilized seeds were germinated on N6CL2 medium (N6 basal, N6 vitamins, sucrose 30 g/l, casamino acid 0.3 g/l, proline 2.8 g/l, 2,4-D 1.0 mg/l, gelrite 4 g/l) for 5 days at 30 °C light and were used as explants for an *Agrobacterium*-mediated transformation. Infected-seedlings were co-cultivated on N6Cl2CO medium (N6 basal, N6 vitamins, sucrose 30 g/l, glucose 10 g/l, casamino acid 0.3 g/l, proline 2.8 g/l, 2,4-D 1.0 mg/l, acetosyryngone 10 mg/l, gelrite 4 g/l) for 3 days at 28 °C in the dark. Co-cultivated seedlings were washed and transferred to N6Cl2TCH25 medium (N6 basal, N6 vitamins, sucrose 30 g/l, casamino acid 0.3 g/l, proline 2.8 g/l, 2,4-D 2.0 mg/l, carbenicillin 0.5 g/l, hygromycin 25 mg/l, gelrite 4 g/l) for 7 days at 30 °C in the light and scutellum tissues were transferred to N6Cl4TCH25 medium (N6 basal, N6 vitamins, sucrose 30 g/l, casamino acid 0.3 g/l, proline 2.8 g/l, 2,4-D 4.0 mg/l, carbenicillin 0.5 g/l, hygromycin 25 mg/l, gelrite 4 g/l) for 7 days at 30 °C in the light. Then, scutellum tissues were cultured on MSRC medium (MS basal, MS vitamins, sucrose 30 g/l, sorbitol 30 g/l, casamino acid 2 g/l, carbenicillin 0.5 g/l, gelrite 4 g/l) at 30 °C in the light. Regenerated shoots were transplanted to MS medium.

2.3. DNA analysis

Genomic DNA samples were prepared from in vitro grown leaves following the supplier's instructions of DNAeasy Plant System (Qiagen) and used as template for PCR analysis. For the GLP-1 analogue gene, two primers were the GLP3-1 primer 5′-ggatccatggctagcaaggtcgtc-3′ and the GLP3-3 5′-gatcactatctcgttgcatgcaacac-3′ (Fig. 1B). PCRs were performed with KOD-Plus polymerase in a 20-μl reaction following the supplier's instructions (TOYOBO, Japan).

2.4. Protein extraction and Western blot analysis

Each mature seed was ground to a fine powder by the Multi-beads shocker (Yasui Kikai, Osaka) as described [17]. Seven hundred microlitres of SDS–urea solution (4% SDS, 8 M urea, 5% 2-mercaptoethanol, 50 mM Tris–HCl (pH 6.8), and 20% (v/v) glycerol) was added to a seed powder and the protein was extracted by vortexing vigorously for 1 h at room temperature. After centrifugation, total protein extracts of the supernatant were applied to SDS–PAGE. Proteins were transferred to a nitrocellulose filter by semidry blotting. The chimeric GLP-1 analogue/globulin proteins were detected by the anti-GLP-1 analogue monoclonal antibody followed by antimouse IgG, coupled to alkaline phosphatase.

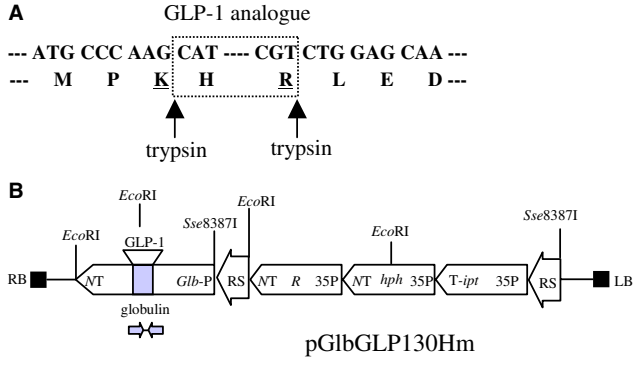

Fig. 1. Schematic structure of the T-DNA of pGlbGLP130Hm used for *Agrobacterium*-mediated rice transformation. (A) Nucleotides and amino acid sequence of the cleavage site for trypsin. The underlined lysine and arginine residues are cleaved with trypsin. (B) The pGlbGLP130Hm plasmid vector for a chimeric GLP-1/globulin. The chimeric GLP-1/globulin gene was cloned into the outside of an excised *R*/RS cassette. The cassette has the *ipt*, *hph* and *R* genes between two directly oriented RS sequence. RB, right border; LB, left border; Glb-P, promoter of rice globulin gene; NT, polyadenylation sequence of nopaline synthase gene; t, polyadenylation sequence of *ipt* gene; NP, promoter of nopaline synthase gene; *ipt*, isopentenyltransferase gene; *hph*, hygromycin phosphotransferase gene; *R*, recombinase gene of yeast site-specific recombination *R*/RS system; RS, recombination sequence of *R*/RS.

2.5. Protein extraction and radioimmunoassay

Mature grains were ground with a mortar and pestle and defatted with chilled acetone. After centrifugation, the pellet was dried up. Then, the chimeric globulin seed storage protein was extracted in 0.025 N NaOH for 24 h at 4 °C. The extracted protein was neutralized with HCl and treated with trypsin for 2 h at 37 °C. The trypsin-digested sample was extracted with ethanol (final concentration, 74.6%). The supernatant was collected and dried up with a centrifuging evaporator and supplied to the radioimmunoassay for quantitative analysis and the in vitro insulin secretion assay. The radioimmunoassay was carried out using by the GLP-1 (Total) RIA Kit (LINCO) according to the supplier's procedure as a standard of [Ser^8, Gln^{26}, Asp^{34}]-GLP-1.

2.6. Assay of insulin secretion

After inoculation in the medium containing 10 mM glucose and the trypsin-digested sample for 45 min at 37 °C, the content of insulin in the medium was measured with the Insulin kit (Shibayagi, AKRIN-011).

3. Results

3.1. Construction of chimeric GLP-1 analogue/globulin protein expression vector

We used a DNA encoding 30 amino acid residues corresponding to peptide [Ser^8, Gln^{26}, Asp^{34}]-GLP-1 (7-36). The GLP-1 analogue gene was inserted into the variable region of the rice 26 kDa globulin gene between residues 109 and 110 (Fig. 1A) [18]. Furthermore, two junction sites of GLP-1 analogue and globulin were replaced by lysine and arginine residues, respectively, for trypsin digestion (Fig. 1A). In the present study, we used the new Hm/MAT-vector into which the *hph* gene and *ipt* gene [19,20] were inserted into the internal region between two directly oriented recombination sequences (RS) (Fig. 1B) [21]. The *hph* gene has been used as a selectable marker gene in addition to the *ipt* gene, and is eliminated from transgenic cells together with the recombinase gene and the *ipt* gene. Therefore, the Hm/MAT-vector can generate selectable marker-free genetically modified rice seeds.

3.2. GLP-1 accumulation in seeds of genetically modified rice

Six independent primary genetically modified rice plants (T_0) were regenerated and cultivated in a greenhouse. These lines were positive when analyzed for the presence of the chimeric GLP-1 analogue/globulin gene by PCR analysis (data not shown). All of them were fertile and seeded. For each T_0 line, ≈10 seeds were horizontally cut into two parts: the grain and the embryo attached to the grain. The accumulation of chimeric GLP-1 analogue/globulin was determined by Western blot analysis in the grains in the T_0 line (Fig. 2). Very high levels of accumulated GLP-1 analogue/globulin were found in all lines (Fig. 2, lanes 1–6).

The grain halves from four genetically modified seeds from each T_0 line were subjected to radioimmunoassay analysis (Fig. 3). No GLP-1 analogue accumulation was detected in the grains of untransformed rice seeds (Fig. 3, lane 7). The highest level of GLP-1 analogue accumulation was observed in line 185-62401 (Fig. 3, lane 1). Independent grains from line 185-62401 accumulated the GLP-1 analogue in amounts ranging from ≈20 to 50 μg/20 mg seed. We also measured, the