Quality and Safety Evaluation of Genetically Engineered Rice with Soybean Glycinin: Analyses of the Grain Composition and Digestibility of Glycinin in Transgenic Rice

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The composition of nutritionally and physiologically important molecules in transgenic rice with the soybean glycinin gene was determined and compared with that of a non-transgenic control. Except for the levels of protein, amino acids and moisture, no marked differences were found between the two kinds of rice. The protein content of the transgenic rice was about 20% higher than the control (control, 6.5 g/100 g; transgenic, 8.0 g/100 g) with a concomitantly lower moisture content. This increased protein content mainly resulted from the increased glycinin expressed in the transgenic rice, and the protein was susceptible to gastric and intestinal digestion juices. In parallel with the increased protein content, some important amino acids lacking in quantity in normal rice were replenished.

Key words: food safety; transgenic rice; composition analysis; soybean glycinin; digestibility

The agri-food industry is now facing the problem of how to control the novel foods being produced by applying biotechnology to enhance the supply of wholesome, nutritious, tasty and affordable foods. With the exception of food additives or some specific foods requiring careful treatment before use, novel foods have so far been introduced to market under the manufacturer’s responsibility. However, this situation is fundamentally now changing with the appearance of biotechnology-derived foods. Several kinds of transgenic crop have been proved to be safe according to the established scientific concepts of “substantial equivalence” and “familiarity,” and some of them have been commercialized. In particular, the production of the glyphosate-tolerant soybeans has rapidly increased in North America. However, the novel biotechnology-derived foods, which are usually designed by introducing genes from edible and non-edible organisms,¹¹ have not been fully accepted by consumers and are required to be assessed in detail regarding their effect on both the environment and health of human beings.¹²

Although, at least in Japan, rice is a traditionally important crop as a staple food, two issues are now being addressed. They are allergenicity and low nutritional quality. The former is mainly caused by a globulin as an allergen, and an individual being allergic to rice must avoid it by scrutinizing its content in food. The elimination of the globulin in rice by a protease treatment is one of the ways to decrease the allergic level of rice.³⁸ An alternative method to repress the expression of the allergen has recently been developed through the construction of transgenic rice by using antisense RNA.⁴ However, an inherent problem is in the low lysine content, one of the essential amino acids, especially for children under 2 years of age.⁵

Glycinin has functions not only to lower the serum cholesterol and blood pressure levels,⁶ but also to induce heat gelation and emulsiﬁcation in food processing.⁸ In order to replenish lysine and the other amino acids lacking in rice, and to provide the required physicochemical and physiological functions, the gene for soybean glycinin (A1aB1b) was inserted between the promoter and terminator of the rice glutation gene by electroproporation into “Matsuyamamii,” an important variety of Japanese rice (Utsumi, et al., submitted for publication). The transgenic rice thus bred is therefore expected to be highly nutritious, in addition to its high processability (Utsumi, et al., submitted for publication). We evaluate here the quality of novel biotechnology-derived rice based on compositional analyses of unpolished rice grains.

Materials and Methods

Rice and culture conditions. Rice (Oriza sativa L., cv Matsuyamamii) was used as a non-transgenic parental control and as the host for the soybean glycinin gene. Seeds of the non-transgenic and transgenic types of rice were sown in pots placed in a closed green house and cultivated for 6 months.

Preparation of crude extracts. Approximately 3 g of unpolished rice was ground with a mortar and pestle in 3 ml of appropriate buffers containing salts or detergents. The homogenate was centrifuged at 13,000 × g for

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Abbreviations: HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SGF, simulated gastric fluid; SIF, simulated intestinal fluid
15 min, and the supernatant was used as a crude extract. Salt-soluble proteins were extracted using a buffer consisting of 35 mM potassium phosphate at pH 7.0, 1 mM EDTA, 0.4 M NaCl and 1 mM phenylmethanesulfonyl fluoride (PhMeSO₂F). Total proteins were extracted with a buffer composed of 50 mM Tris-HCl at pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS) and 2% mercaptoethanol.

Protein concentration. Protein was determined by the method of Lowry et al.,19 with bovine serum albumin being used as a standard.

Electrophoresis and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli,10 this being followed by Western blotting,11 except for the use of a peroxidase-labeled secondary antibody.

Analytical assays. Compositional analyses of the unpolished rice samples were performed at Japan Food Research Laboratories (Osaka, Japan). After milling a sample, the resultant powder was subjected to the analyses. Almost all of the compositional values were determined by 2 or 4 experiments.

(i) Moisture. Moisture was determined by weight loss on drying.
(ii) Protein. Total nitrogen was determined by the Kjeldahl method, protein then being calculated from the total nitrogen by using N × 6.25.
(iii) Lipid. Lipid was determined by AOAC official method 922.06.12
(iv) Fiber. Fiber was determined by the modified Henneberg-Stohmann method.13
(v) Ash. The sample was charred and ashed to a constant weight, the residue being quantified as ash.
(vi) Carbohydrate. Carbohydrate was calculated by the following equation: carbohydrate (g/100 g) = 100 - (moisture + protein + lipid + fiber + ash) g/100 g.
(vii) Minerals. Phosphorus and iron were determined by vanadomolybdate absorption and phenanthroline absorption analyses, respectively. Calcium, sodium, potassium, and magnesium were determined by atomic absorption analyses.
(viii) Vitamins. Thiamine-HCl and riboflavin were determined by high-performance liquid chromatography (HPLC). Vitamin B₆ and niacin were bioassayed by using Saccharomyces cerevisiae ATCC9080 and Lactobacillus plantarum ATCC8014, respectively.
(ix) Amino acids. A sample was hydrolyzed under vacuum with HCl, neutralized, and then subjected to an automated amino acid analysis. Methionine and cystine were oxidized with performic acid, hydrolyzed with HCl, and analyzed as already described. Tryptophan was determined by HPLC.
(x) Fatty acids. The profile of the fatty acids was calculated by gas chromatography.

Determination of expressed glycinin. The expression level of glycinin in the rice samples was determined by dot-blotting as described previously11 and by an analysis of the image profile on the PAGE gel with the NIH Image 1.59 program.

In vitro digestion of glycinin. The crude extract from the transgenic rice was incubated at 30°C with simulated gastric (SGF) and intestinal fluids (SIF)10 and the reaction was periodically stopped by heating at 95°C. The reaction mixture was subjected to SDS-PAGE and then to Western blotting as already described.

Results
Shape of the transgenic rice grains
There were no significant differences in the appearance and weight between the non-transgenic and transgenic rice grains (Fig. 1). The grains used for the subsequent experiments were about 6 mm in length × 3 mm and about 23 mg in weight.

Expression level of soybean glycinin in rice
Although glycinin was undetectable in the control (Fig. 2A, lane 1), the expression of glycinin in the transgenic rice was clearly observed (Fig. 2A, lane 2). In contrast, the non-transgenic rice (Fig. 2B) did not show any detectable expression of glycinin.

Fig. 1. Rice Samples Used for the Safety Assessment. A, control; B, transgenic. The bar indicates 2 cm in length.

Fig. 2. Electrophoretic Profile of Rice Protein Revealed by Immuno-Staining (A) and Protein Staining (B). Lane 1, salt-soluble proteins in the control rice; lane 2, salt-soluble proteins in the transgenic rice; 3, total proteins in the control rice; 4, total proteins in the transgenic rice. P and Ac indicate the precursor and acidic subunit of glycinin, respectively. An asterisk “*” indicates the 32 kDa protein absent from the transgenic rice.
The amino acid composition of the rice samples was analyzed (Table 2). The content of each amino acid was expressed as g/100 g ± SEM.

The fatty acid composition of the rice samples was also analyzed (Table 3). The content of each fatty acid was expressed as g/100 g.

The vitamin composition of the rice samples was analyzed (Table 4). The content of each vitamin was expressed as mg/100 g ± SEM.
Table 5. Mineral Composition of the Rice Samples

<table>
<thead>
<tr>
<th>Rice</th>
<th>Phosphorous (mg/100g±SEM)</th>
<th>Iron (mg/100g±SEM)</th>
<th>Calcium (mg/100g±SEM)</th>
<th>Sodium (mg/100g±SEM)</th>
<th>Potassium (mg/100g±SEM)</th>
<th>Magnesium (mg/100g±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>288±5.67</td>
<td>1.15±0.07</td>
<td>11.0±0.34</td>
<td>2.2±0.07</td>
<td>251±6.75</td>
<td>99.0±0.70</td>
</tr>
<tr>
<td>Transgenic</td>
<td>279±4.23</td>
<td>1.12±0.01</td>
<td>12.1±0.35</td>
<td>2.4±0.10</td>
<td>256±3.25</td>
<td>106±0.85</td>
</tr>
</tbody>
</table>

Each value is the mean±SEM.

Fig. 3. Digestibility of Glycinin Expressed in the Transgenic Rice.

The crude extract (10 µg of protein) from the transgenic rice was incubated at 30°C with simulated gastric (SGF) and intestinal (SIF) fluids. At the prescribed time, the sample was taken and subjected to SDS-PAGE, this being followed by protein staining (A) and immuno-staining (B). Lane 1, 0 min (SGF); lane 2, 10 min (SGF); lane 3, 0 min (SIF); lane 4, 30 min (SIF). P and Ac indicate the precursor and acidic subunit of glycinin, respectively.

of minerals and vitamins between the control and transgenic rice samples, except for vitamin B₆, which was appreciably higher (50% more) in the transgenic rice (Tables 4 and 5).

(v) Digestibility of the expressed glycinin

The digestibility of the expressed glycinin in the transgenic rice was confirmed by using simulated gastric (SGF) and intestinal (SIF) fluids. Protein staining (Fig. 3A) and immuno-staining (Fig. 3B) indicated that high-molecular-weight proteins, including glycinin, were almost completely digested within 10 min in SGF. Glycinin was completely degraded within 30 min in SIF. Although undigestible proteins (especially the 50 kDa protein) by SIF were apparent after the SIF treatment, they were the SIF-resistant proteins inherently contained in rice.

Discussion

The quality of genetically engineered rice with the soybean glycinin gene was assessed by analyzing the components. Analyses were carried out with regard to about 60 components that are the nutritionally and physiologically important classes of compounds in rice. The results obtained for the control rice were comparable with those previously reported for rice; the compositional analyses made on the transgenic rice with the soybean glycinin gene are, therefore, thought to be reliable.

Based on the notion that novel biotechnology-derived foods should be at least as safe as traditional foods having a long history of safe use, the concept of "substantial equivalence" was established and adapted to the safety assessment of novel biotechnology-derived foods. Utilization of this concept allows the nature and extent of the required safety to be assessed. "Substantial equivalence" may be classified in three different ways: (i) Substantially equivalent: this implies complete biochemical identity between the novel food and the existing food, except for the properties conferred by the introduced genes. (ii) Substantially equivalent except for some defined traits: the novel food is substantially equivalent to its traditional counterpart, except for certain identifiable and defined differences, such that the differing properties would require specific safety studies. (iii) Lack of equivalence: the novel food differs from the traditional product in multiple undefined respects, such that the novel food would need to be thoroughly examined to assess its safety, before accepted or rejected.

The transgenic rice with the glycinin gene presented here differs from the non-transgenic control in the amounts of protein (Table 1) and vitamin B₆ (Table 4) that are present. The amount of protein in the transgenic rice is 1.2-fold higher than that in control, and the increase was possibly caused by the expression of glycinin. The higher level of protein is thought not to be harmful, since it was readily susceptible to digestive juices (Fig. 3). The higher levels of vitamin B₆ and a few fatty acids in the transgenic rice seemed not to affect the health of human beings, because the excess water-soluble vitamin B₆ was discharged and the levels of the fatty acids were not much higher than normal. Therefore, the safety of the transgenic rice may be guaranteed by "substantial equivalence" according to above-mentioned classification (ii).

As to the reasons for the different levels of protein, vitamins and fatty acids in the transgenic rice presented here, two are considered plausible. (a) Position effect: When new genes are inserted into any organism, there is a "position effect" which entails an unpredictable pattern of gene expression and genetic function. The insertion of genes may disrupt certain genes or, in combination with the inserted gene products, may induce the expression of silent genes. (b) Metabolic interference: Inserted gene products may interfere with the metabolic pathway through the interaction with enzymes on the metabolic pathway which would bring about an accumulation or disappearance of metabolites in the host cells. Whatever the reason for the different cellular components in transgenic rice, it cannot be disregarded that the
metabolism of the host cells would be significantly disturbed by the inserted genes and/or their products, since the protein product of the activated gene may induce unexpected reactions and produce potentially toxic products, and metabolic modulation may result in the accumulation hazardous compounds as was indicated in the case of recombinant yeast\textsuperscript{16} and transgenic potatoes (Hashimoto et al., unpublished results). Thus, the inserted gene, even in a very simple organism such as a bacterium, may act differently when working within its new host, so that the original genetic intelligence of the host will be disrupted. Therefore, before introducing genetically altered crops to the market, more attention should be paid to possible un-expected and un-desirable metabolic disturbances caused by the inserted genes.

The transgenic rice constructed by using the soybean glycamin gene is considered to be highly nutritive, physiologically significant and processable. Such valuable properties of transgenic rice may be useful for improving or maintaining health, especially for older people, and for enhancing the rice manufacturing processes. To confirm the safety of transgenic rice presented here, more detailed nutritional and toxicological studies are now being carried out in parallel with the development of a monitoring method for the novel rice which is similar to that used for transgenic soybeans.\textsuperscript{16}

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References