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Safety assessment of genetically modified rice expressing human serum albumin from urine metabonomics and fecal bacterial profile

Xiaozhe Qi [a], Siyuan Chen [a], Yao Sheng [a], Mingzhang Guo [a], Yifei Liu [a], Xiaoyun He [a,b], Kunlun Huang [a,b], Wentao Xu [a,b,*]

[a] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] The Supervision, Inspection and Testing Center of Genetically Modified Organisms, Ministry of Agriculture, Beijing 100083, China

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ABSTRACT

The genetically modified (GM) rice expressing human serum albumin (HSA) is used for non-food purposes; however, its food safety assessment should be conducted due to the probability of accidental mixture with conventional food. In this research, Sprague Dawley rats were fed diets containing 50% (wt/wt) GM rice expressing HSA or non-GM rice for 90 days. Urine metabolites were detected by ^{1}H NMR to examine the changes of the metabolites in the dynamic process of metabolism. Fecal bacterial profiles were detected by denaturing gradient gel electrophoresis to reflect intestinal health. Additionally, short chain fatty acids and fecal enzymes were investigated. The results showed that compared with rats fed the non-GM rice, some significant differences were observed in rats fed with the GM rice; however, these changes were not significantly different from the control diet group. Additionally, the gut microbiota was associated with blood indexes and urine metabolites. In conclusion, the GM rice diet is as safe as the traditional daily diet. Furthermore, urine metabonomics and fecal bacterial profiles provide a non-invasive food safety assessment rat model for genetically modified crops that are used for non-food/feed purposes. Fecal bacterial profiles have the potential for predicting the change of blood indexes in future.

1. Introduction

Modern techniques of genetic engineering have been used to introduce specific genetic material derived from any species of plant, animal, microorganism or even synthetic material into different species of plants by altering the genetic material (Zhang and Shi, 2011). After such genetic alteration, the resulting plants can express novel and desirable traits, such as enhanced disease resistance and anti-stress characteristics. In recent years, a third generation of genetically modified (GM) crops has risen up, called "Pharma plants" (Alderborn et al., 2010). Such plants are used as heterologous expression platforms for recombinant proteins, including native and modified therapeutic proteins. "Pharma plants" can produce drugs, such as antibodies and vaccines, to prevent and cure diseases (Pandey et al., 2010). Plants have been selected to express the proteins for drug and industry use because they have several advantages over the traditional platforms for recombinant protein production; they are inexpensive, highly scalable and do not support human pathogens (Paul and Ma, 2011). Crops producing pharmaceutical proteins can be established with minimal upfront investment in infrastructure, unlike the major fermentation-based platforms (Fischer et al., 2012).

Human serum albumin (HSA) functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a pivotal role in the stabilization of extracellular fluid volume. HSA has been widely used clinically to treat serious burn injuries, hemorrhagic shock, hypoproteinemia, fetal erythroblastosis, and ascites caused by cirrhosis of the liver (Hastings and Wolf, 1992). In addition, HSA is used as an excipient for vaccines or therapeutic protein drugs as well as a cell culture medium supplement (Marth and Kleinhappl, 2001). Approximately more than 500 tons of HSA is produced each year worldwide. Currently, globally commercial HSA production is primarily based on collected human plasma, which is limited in supply, leading to an increase in price. Furthermore, there is a potential risk of contact with blood-derived infectious

Abbreviations: GM, genetically modified; HSA, human serum albumin; NMR, nuclear magnetic resonance; DGGE, denaturing gradient gel electrophoresis; SCFA, short chain fatty acids; PLS, partial least squares; PCA, principal component analysis; WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; PLT, platelet count; MPV, mean platelet volume; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein; ALB, albumin; ALP, alkaline phosphatase; GLU, glucose; BUN, blood urea nitrogen; CREA, creatinine; Ca, calcium; P, phosphorus; CHO, total cholesterol; LDH, lactate dehydrogenase; Cl, chlorine; Mg, magnesium.

* Corresponding author. College of Food Science and Nutritional Engineering, China Agricultural University, No. 17 Tsinghua Donglu, Beijing 100083, China. Tel.: +86 (8610) 62738793; fax: +86 (8610) 62738793.
E-mail address: xuwentao@cau.edu.cn (W. Xu).

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pathogens during the process of utilizing plasma-derived HSA (Chamberland et al., 2001). Due to the above limitations, He et al. developed GM rice that can stably express recombinant HSA (rHSA) (He et al., 2011); rHSA can be produced on large scale and we can avoid the potential abovementioned problems. Although the GM rice is for non-food purposes, its food safety assessment should be conducted because several episodes of accidental mixture of conventional food plants with GM food plants have been reported, such as the StarLink™ maize event (Lu, 2008), herbicide-resistant Liberty Link 601 transgene rice (Li, 2008), and Bt63 rice mixing with food on the EU market without being authorized (Holst-Jensen, 2008). We cannot guarantee that the GM rice expressing HSA for non-food purposes would not be mixed with the rice for food, resulting in accidental consumption. Therefore, food safety assessment for the GM rice expressing HSA is necessary.

Analyses for the metabolites excreted in urine and feces are supplements for traditional methods for safety assessment of GM food. Metabolites, in the context of metabolomics, are small molecules, naturally occurring in cells, fluids and excreta of living organisms; hence in this context they are not breakdown products of exogenous chemicals. They are regarded as simple to conduct, non-time-consuming, low cost and do not cause damage to experimental animals compared with traditional methods. Traditional safety assessment for GM products as whole food involved food consumption, hematology, blood chemistry, organ weight, and gross histopathological examination, which are targetly investigated. This approach is primarily used as the first step to assess the safety of genetically modified crops. Urine and fecal metabolites can reflect unintended effects. Potential unintended side effects should be considered before GM plants enter the market; however, the approach has not been applied in the assessment. Nuclear magnetic resonance (NMR) can be used in improving our understanding of diseases and mechanisms in support of toxicology and pathology investigations in preclinical drug development (Clarke and Haselden, 2008). Previous studies have used NMR to study the food safety of genetically modified rice (Cao et al., 2011, 2012). Additionally, gut is the first defensive line to protect the body from outside damage. Bacterial profiles are influenced by the host genotype, diet, age, and sex. The observed host metabolic phenotype is strongly influenced by the gut microbiome. Denaturing gradient gel electrophoresis (DGGE) is often used to analyze microbial composition in the gut (Queipo-Ortuno et al., 2013; Xie et al., 2012). Diversity of the ileum and cecum microflora was determined by DGGE technique to analyze the effects of GM soybeans on the intestinal microflora of broiler chickens (Tan et al., 2012). If the transgenic soybean meal is harmful to broilers, it may first damage the intestinal protection system, especially of the intestinal mucosal immune system and the intestinal microflora of broilers. What more, Cao et al. (2012) used the DGGE method to detect the fecal bacterial profiles of rats fed with GM rice. The result was comparable with the results attained using the traditional method. Urine and feces can be acquired without damaging the animal. In addition, the results are less influenced by experimental manipulation, such as blood collection and dissection. Currently, no standards in regards to food safety assessment have been established for pharmaceutical GM crops. We are the first to use the metabolite profile of rat urine and feces to evaluate the food safety of GM rice expressing HSA. In addition, we constructed a metabolite model for the food safety assessment of GM crops for non-food purposes.

In this study, Sprague-Dawley rats were fed diets containing 50% (wt/wt) GM rice. This study was conducted in compliance with Chinese Toxicology Assessment Procedures and Methods for Food Safety (GB15193.13, 2003, C. S., 2003). A novel assessment approach is constructed. The urine metabonomics and bacterial profiles were analyzed at two time points.

2. Materials and methods

2.1. Ethics statement

Animal studies were conducted in accordance with the ethical guidelines for the care and use of laboratory animals of the Supervision & Testing Center for Genetically Modified Organisms Food Safety, Ministry of Agriculture (Beijing, China). All procedures were approved by the Animal Care Ethics Committee of China Agricultural University.

2.2. Materials

GM 4-114-7 rice and its non-transgenic parent "Taipei 309" were provided by Wuhan University, China. Both rice strains were planted in Taoxian City, Hubei Province, China in identical climate conditions in 2011. The rice grains were dehulled and stored at 4 °C. Analyses of nutritional proximates (moisture, ash, crude protein, fat, and crude fiber), minerals, amino acids, and anti-nutritional factors of the GM rice and the non-GM rice were conducted in accordance with Chinese standard methods (Sheng et al., 2014). The GM rice 4-114-7 and its non-transgenic parent "Taipei 309" brown rice were used at 50% wt/wt (Domon et al., 2009; Schroder et al., 2007; Zhou et al., 2011) to produce diets comparable to the commercialized rodent diet by the Ke Ao Xie Li Feed Co. (Beijing, China).

2.3. Animals

Four-week-old Sprague-Dawley male and female rats were obtained from Vital River Laboratories Inc. (Beijing, China). After acclimatization for five days, rats were randomly divided into three groups (10 rats/sex/group). The three groups were respectively administered diets with 50% (wt/wt) GM rice, diets with 50% (wt/wt) non-GM rice or commercialized rodent diet. The animals were housed (5 rats/cage) in a specific pathogen-free animal laboratory at the Supervision & Testing Center for GMOs Food Safety, Ministry of Agriculture in standard laboratory conditions (adequate fresh air exchange, temperature 20–25 °C and relative humidity 40–70%). A 12-h light/dark automatic cycle of artificial illumination was used. Rats were provided water and feed *ad libitum*. This study was approved by the Animal Ethics Committee of China Agricultural University with the approval ID 20120011.

2.4. Urine sampling

Urine samples were collected from the rats in each group (10 rats/sex/group) for 24 h on days 30 and 90 (Cao et al., 2012).

2.5. Urine assay

A urinalysis is an array of tests performed on urine, including ketones, bilirubin, protein, glucose, urine specific gravity, occult blood, pH, bacteria, erythrocytes, white blood cells, squamous epithelium and clarity, tested by an IRIS IQ200 Elite Urine Analyzer (USA).

2.6. NMR spectroscopy

Urine samples were prepared as previously described (Cao et al., 2011). Phosphate buffer solution (350 μL; pH 7.4) was mixed with urine (350 μL) to provide stabilization of the urinary pH. The samples were centrifuged at 12,000 *g* for 10 min to remove any insoluble materials. Then, 30 μL TSP/D_2O (1 mg/mL) was mixed with the 600 μL supernatants from each sample. The samples were transferred to 5 mm NMR tubes and analyzed using a Unity-Inova 600 superconducting NMR spectrometer (USA). Data obtained from the NMR were analyzed by SIMCA-P + (version 10.04, Umetrics, Umea, Sweden), and the results were shown by scores plot and loadings plot (Cui et al., 2013).

2.7. DNA extraction from feces

Approximately 0.2 g of fecal sample was collected for DNA extraction using a stool extraction mini kit (Qiagen, Germany) on days 30 and 90, according to manufacturer's instructions. OD260/280 DNA ratios ranging from 1.8 to 2.0 were selected for quantification (Yuan et al., 2013).

2.8. Denaturing gradient gel electrophoresis method for analyses of the bacterial profile

The V3 region of the 16S rRNA gene for all bacteria was amplified with primers 518R and GC-338F (Yuan et al., 2013). Denaturing gradient gel electrophoresis was performed using the Dcode™ Universal Mutation Detection System (BioRad). A denaturing gradient of 40–60% was prepared with the gradient delivery system (Model 475, Bio-Rad). Band simulation was conducted using Quantity One software (Bio-Rad). Then, clustering as well as diversity analysis were performed by PAST (Hammer