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Long-term toxicity study on transgenic rice with *Cry1Ac* and *sck* genes

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ABSTRACT

In the present work, we evaluated the chronic effects of the transgenic insect-resistant rice carrying *Cry1Ac* and *sck* genes on Sprague–Dawley (SD) rats through a 78-week feeding study. Based on the gender and weight, 180 SD rats were randomly and evenly assigned into three groups. GM rice and non-GM rice were separately formulated into diets at high levels. AIN-93 diet was used as a nutritional control. Body weight, food consumption, hematology and serum chemistry were monitored regularly. Rats were sacrificed for organ weight measurement and pathological examination at 52 weeks and 78 weeks. Body weight, food consumption, mortality rates, tumor incidences and pathological findings showed no significant difference among the three groups. Although certain differences in some hematology, serum chemistry parameters and relative organ weights were observed between GM rice group and control groups, they were not considered as treatment-related. Taken together, long-term intake of transgenic rice carrying *Cry1Ac* and *sck* genes at a high level exerts no unintended adverse effects on rats.

1. Introduction

Cultivated in more than 100 countries worldwide, rice is a staple food source for about a half of the global population. Asia is the main producer of rice, and China is the country with the highest rice production (OECD, 2004). With the rapid development of genetically modified (GM) food throughout the world, a number of transgenic rice plants have been produced with traits like herbicide, biotic stress and abiotic stress tolerance. In addition, traits of increased nutritional value have also been introduced into rice, such as golden rice which is enriched with vitamin A (Bajaj and Mohanty, 2005; Kathuria et al., 2007). Since rice is one of the most important cereal crops, safety assessment of transgenic rice should be particularly stringent before its commercialization. So far, no transgenic rice has been approved for commercial cultivation (He et al., 2008).

In this study, the transgenic rice carrying two insecticidal genes (*Cry1Ac and sck* genes) was developed by Institute of Genetics and Developmental Biology, Chinese Academy of Sciences in collaboration with Fujian Academy of Agricultural Sciences through an *Agrobacterium*-mediated transformation method. Moreover, its parent rice, an indica rice restorer line named Minghui 86 (M86), was used as the control. The cultivation process has been described by previous works (Zhou et al., 2012). M86 was used as the host and a parental control. The *sck* (Signal-CpTI-KDEL) gene is a modified cowpea trypsin inhibitor (CpTI) gene to increase the stability of CpTI protein, which is generated by fusing a signal peptide sequence at the CpTI 5′ end and an endoplasm reticulum retention signal peptide at the 3′ end, respectively (Deng et al., 2003). The transgenic rice combining different insecticidal mechanisms of *Cry1Ac* and *sck* genes exhibits higher resistance than GM rice transformed with single *sck* gene (Zhang et al., 2007). Some investigations have been carried out to assess the safety of the transgenic rice, and no adverse effects have been observed (Qin et al., 2012; Xu et al., 2011). Based on these results, no evidence indicated that the transgenic rice has adverse effects.

Ninety-day feeding studies in rodents have been recommended as one of the primary assessment procedures for GM crops (FAO/WHO, 2000; EFSA, 2008). Recently, a long-term feeding study found that rats fed with glyphosate-resistant maize for 2 years develop more tumors and die earlier than control (Séralini et al., 2012). However, many scientists pointed out many errors and inaccuracies in this study, such as poor study design, inadequate statistical analysis and misleading conclusions (Arjó et al., 2013). Moreover, GM crops have no adverse effects on SD rats according to two chronic feeding studies of GM soybeans (52-week and 104-week) and a 52-week feeding study of GM rice expressing human lactoferrin (Sakamoto et al., 2007, 2008; Zhang et al., 2012).

Abbreviations: EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization of the United Nations; GM, genetically modified; OECD, Organization for Economic Cooperation and Development; SD rats, Sprague–Dawley rats; SPSS, statistics package for social science; WHO, World Health Organization.

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The subchronic feeding study cannot detect effects on reproduction or development (EFSA, 2008). Some researchers have proposed that it is insufficient to evaluate chronic toxicity through 90-day-long tests, and long-term and multigenerational studies ought to be conducted (Séralini et al., 2011). Snell et al. (2012) stated that chronic and multigenerational studies should be conducted in a case-by-case approach if some reasonable doubt remains after a 90-day feeding trails. So far, few long-term feeding studies have been performed on GM crops. Transgenic products, which could act as staples (such as rice and wheat), have higher consumption exposure than other GM crops and consumers will pay more attention to the safety of these GM products. After all, the duration of Subchronic study (3 months) is only 1/7 to 1/8 of the life span of rats. Such question will be raised by consumers: "It might be safe to use the GM rice for several years. However, its long-term safety is not guaranteed?" Therefore, it is necessary to carry out the long-term feeding studies on these GM foods in order to ease the concerns of consumers and provide more detailed scientific and reliable evidences for the safety of GM foods.

In this study, we performed a 78-week feeding experiment with transgenic rice at high levels in diets and evaluated the potential adverse effects of the transgenic rice carrying *Cry1Ac* and *sck* genes on rats. In addition, this study was conducted in accordance with OECD Test Guideline 452: Chronic Toxicity Studies, (OECD, 2008) and OECD Good Laboratory Practice guidelines.

2. Materials and methods

2.1. Plant materials

The insect-resistant rice carrying *Cry1Ac* and *sck* genes and its parental control (M86) were both simultaneously cultivated in two adjoining plots in the experimental field of Fujian Academy of Agricultural Sciences under identical environmental conditions. The presence of the *Cry1Ac* and *sck* transformation cassette was confirmed by PCR using standard protocols. Transgene expression of Cry1Ac and CpTI in mature seeds was verified by ELISA assay. Cry1Ac was detected by a commercially available kit (Envirologix Inc., USA). CpTI was detected using a previously described method (Wang et al., 2005). The Cry1Ac content was about 6.1 μg per gram rice, and the CpTI content was below 0.035 μg per gram (the detectable threshold).

2.2. Composition analysis of rice and diet formulation

Nutritional proximates (protein, fat, moisture, ash and fiber) of GM rice and non-GM rice were analyzed according to standard methods (Chinese Standard GB 5009. 3–5-2010; GB/T 5009. 6-2003; GB/T 5009. 88-2008). Carbohydrate levels were estimated by the formulation as follows.

$$\%\text{carbohydrate} = 100 - (\%\text{protein} + \%\text{fat} + \%\text{ash} + \%\text{moisture})$$

Based on the nutritional components of the two types of rice and AIN-93 guidelines, AIN-93G for growth phase of rats (1–13 weeks) and AIN-93 M for maintenance of adult rats (14–78 weeks), established by the American Institute of Nutrition (Reeves et al., 1993), flours from GM rice and non-GM rice were formulated into rodent diets at concentrations of 73.2% and 73.8% respectively for the growth phase, while it was 81.5% and 82.2% for the maintenance phase, respectively. AIN-93 purified diet was used as an additional negative control. All diets were pelleted by Beijing HFK Bioscience Co., Ltd., (Beijing, China), then vacuum-packed and sterilized by ^{60}Co. Table 2 lists the formulation of diets for growth and adult maintenance phases as well as the results of nutritional analysis of complete feeds.

2.3. Animals and housing conditions

A total of 100 male and 100 female weaning 4-week-old SD rats, with an average body weight of 40–50 g, were obtained from the Experimental Animal Center of Health Science Center, Peking University (Beijing, China) [license number: SCXK (JING) 2006-0008 and SCXK (JING) 2006-0025]. Animals were housed in an environmentally controlled breeding room (temperature of 24 ± 2 °C; humidity of 50 ± 10%) illuminated by artificial light with a 12-h light/dark cycle. After a 5-day acclimation, 90 male and 90 female healthy rats were selected, and they were then randomly and evenly assigned into three groups as follows: GM rice group (group A), non-GM rice group (group B) and AIN-93 control group (group C). All rats were individually housed and provided with diet and filtered tap water *ad libitum*.

2.4. Clinical observations, body weight and food consumption

All animals were daily monitored for abnormalities and mortality. Body weight of each animal was recorded once a week for the first 13 weeks, and monthly thereafter. Food consumption for each animal was determined twice a week for the first 13 weeks, and weekly thereafter.

2.5. Hematology

Hematological examinations were carried out at 13, 26, 52 and 78 weeks. Briefly, 10 male and 10 female rats were randomly selected from each group and fasted for 16 h. Blood was collected via the tail vein and placed in tubes containing anticoagulant. White blood cell (WBC) count, red blood cell (RBC) count, hemoglobin concentration (HGB), platelet (PLT) count, lymphocyte percentage (LYM%), intermediate cell (MID%) and neutrophilic granulocyte (GRN%) were determined with a MEK-6138K Hematology Analyzer (Nihon Kohden, Japan).

2.6. Serum chemistry

For serum chemistry analyses, blood was collected into tubes without anticoagulant at the same time intervals of the hematological investigations, and serum was obtained through centrifugation. Serum chemistry parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), alkaline phosphatase (ALP), glucose (GLU), urea nitrogen (BUN), creatinine (CRE), total cholesterol (CHO), triglycerides (TG) and lactate dehydrogenase (LDH), were determined with an automatic Biochemical Analyzer (Autolab, Italy).

2.7. Pathology

In order to obtain the information on the progression of toxicological changes, 10 rats/sex/group were randomly selected to euthanize at 52 weeks. Selected rats were fasted for 16 h, then weighed and anesthetized with 10% choral hydrate. Subsequently, animals were sacrificed by exsanguination, and a full, detailed gross necropsy was followed. Organs, including heart, liver, spleen, kidneys, adrenals, thymus, testes, epididymides, uterus, ovaries and brain, were dissected and weighed as soon as possible. Paired organs were weighed together. The relative organ weight was expressed as a percentage of the final individual body weight. At the end of the experiment (78 weeks), all surviving rats were sacrificed with the method mentioned above.

In addition to above organs, pituitary, thyroid, parathyroid, trachea, lung, stomach, duodenum, jejunum, ileum, colon, caecum, rectum, pancreas, prostate, urinary bladder, lymph nodes and suspected tissues were also sampled and preserved in 10% neutral buffered formalin for the subsequent histopathological examination.

2.8. Statistical analysis

Quantitative data were presented as mean values ± standard deviation ($\bar{x} \pm s$). Homogeneity variance was analyzed using Levenes's test firstly. Homogeneity variance was analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test, while non-homogeneity variance was analyzed after data transformations. Qualitative data (such as mortality rate and tumor incidence) were analyzed using chi-square test. Kaplan–Meier method and log-rank test were used for the survival analysis. Statistical analyses were carried out using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Composition analysis

Table 1 lists the main nutritional compositions of GM rice and non-GM rice. We did not observe significant differences between these two rice types. Table 2 shows the nutritional analysis data

Table 1
Nutritional components of transgenic and conventional rice flour (%).

Nutrients	GM rice	Non-GM rice
Protein	6.97	6.87
Fat	1.1	1.1
Moisture	10.6	12.2
Ash	0.44	0.49
Carbohydrate	80.9	79.4
Fiber	2.27	2.70