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ScienceDirect
Food and Chemical Toxicology 45 (2007) 364–377
www.elsevier.com/locate/foodchemtox

Safety testing of GM-rice expressing PHA-E lectin using a new animal test design

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Received 14 October 2005; accepted 1 September 2006

Abstract

The 90-day animal study is the core study for the safety assessment of genetically modified foods in the SAFOTEST project. The model compound tested in the 90-day study was a rice variety expressing the kidney bean *Phaseolus vulgaris* lectin agglutinin E-form (PHA-E lectin). Female Wistar rats were given a nutritionally balanced purified diet with 60% parental rice, 60% PHA-E rice or 60% PHA-E rice spiked with 0.1% recombinant PHA-E lectin for 90 days. This corresponded to a mean daily PHA-E lectin intake of approximately 0, 30 and 100 mg/kg body weight for each group, respectively. The spiking was used to increase the specificity and to demonstrate the sensitivity of the study. A range of biological, biochemical, microbiological and pathological parameters were examined and significant differences in weight of small intestine, stomach and pancreas and plasma biochemistry were seen between groups. Included in this paper are also data from the molecular characterisation and chemical analysis of the PHA-E rice, from the construction and production of the PHA-E lectin, and from the preceding 28-day *in vivo* study where the toxicity of the pure PHA-E lectin was determined. In conclusion, the combined use of information from the compositional analysis, the 28-day study and the characterisation of the PHA-E rice and the PHA-E lectin has improved the design of the 90-day study. The spiking procedure has facilitated the interpretation of the results of the study and transferred it into a valuable tool for the future safety testing of genetically modified foods.

Keywords: Genetically modified rice; GMO; Safety assessment; Animal study; PHA-E lectin; Spiking; SAFOTEST

1. Introduction

In Europe, methodologies for safety assessments of genetically modified (GM) foods and derived products are not harmonized. Recommendations of the EU Commission indicated that the difficulty to use the traditionally designed animal feeding studies like the OECD guideline tests for toxicological assessments remains as a major challenge in the risk assessment strategies of GM foods (European Commission, 1997). Therefore, validated sensitive and specific nutritional-toxicological testing procedures *in vivo* and *in vitro* for the safety assessment of GM food were urgently

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doi:10.1016/j.fct.2006.09.003

requested. As a consequence, a EU-project entitled "New methods for the safety testing of transgenic food" (SAFOTEST) funded by the Fifth Framework Programme for Research was initiated in February 2000 with the overall objective to develop and to validate the scientific methodology, which is necessary for assessing the safety of food from genetically modified plants.

In order to evaluate the *in vivo* testing approach proposed within the project a transgenic rice with inserted copies of a gene encoding an insecticidal protein from the kidney bean: the lectin *Phaseolus vulgaris* agglutinin E-form (PHA-E lectin), which is known to possess high mammalian toxicity was developed (Leavitt et al., 1977). The PHA-E expressing rice was constructed, characterised and bulked solely to be tested in this project as a model rice with a potential for leading to mammalian toxicity when tested in its raw uncooked form. In addition, recombinant PHA-E lectin was expressed in the yeast *Pichia pastoris* and purified and characterised for later usage as spiking material in the *in vivo* studies.

The project design recognised that testing of whole foods in experimental animals presents a scientific and technical challenge as large quantities of the whole food need to be administered with the possibility of dietary imbalance of the animal feed. Sometimes only small margins of safety are achieved compared to the testing of single chemical substances.

The purpose of the present study was therefore to perform a 90-day study with an improved sensitivity and specificity compared to the traditional 90-day feeding study (OECD guideline study no. 408).

The 90-day study in SAFOTEST represented the second step in a two-step safety testing procedure. The first step in the safety testing procedure comprised the molecular characterisation, the chemical analyses of the PHA-E rice and the parental control rice as well as performance of short-term *in vitro* and *in vivo* studies, all contributing to the design of the core 90-day study at the second step.

Data in the present paper includes results from the molecular characterisation, chemical analyses of the rice, and construction and production of the recombinant PHA-E lectin. In addition, results are presented from the 28-day *in vivo* study where the toxicity of the pure PHA-E lectin is determined as well as the results from the core 90-day feeding study where the safety of the whole GM rice is tested.

2. Materials and methods

2.1. Production and characterisation of PHA-E expressing seeds for use as test material

2.1.1. Rice transformation

Primary transformants of rice plants (*Oryza sativa* L. variety EYI105) containing the gene construct for expression of PHA-E (*P. vulgaris* agglutinin E-form) were generated by particle gun bombardment of mature embryos as previously described (Sudhakar et al., 1998); two DNA plasmids, pUbiPHA-E and pWRG1515 (contains both the hygromycin–resistance gene, *hpt*, and the reporter gene *gusA*), at a molar ratio of 3:1, were used simultaneously in the bombardment. Following co-transformation, calli were transferred to a proliferation medium to allow plantlet formation, and transformants were selected on the basis of hygromycin-resistance. Putative transformants were screened for the presence of the transgene by PCR. Previous experiments have shown that a high frequency (>75%) of selected plantlets will show co-transformation of the selectable marker and the desired transgene. Rooted plantlets grown in soil were then screened for the presence and accumulation of PHA-E by immuno-assay plants. On the basis of the level of PHA-E expression, line 35 was selected for bulking up in the field.

2.1.2. Bulking up in the field

Rice seeds of both the transgenic and the parental variety EYI105 to be used in the animal studies were grown in the Experimental Farm of Zhejiang University at Jiande County, Zhejiang Province of China. Seeds of PCR-positive T1 plants of PHA-E rice line 35 were harvested and further bulked up for two generations in the field to produce sufficient quantities of seed material for animal feeding studies; and consequently seeds of PHA-E rice at T4 generation were used in animal diets. Rice seeds of both transgenic and parent were sown at the same date and field, and 30 days after sowing, seedlings were transplanted to another field side by side. Regular practice of field management, fertilizer application and pest control were applied. Rice seeds were harvested in about 4 weeks after heading. The rice seeds were later sent from China to Denmark as whole rough rice.

2.1.3. Quantification of transgene expression

The levels of PHA-E expression and accumulation were quantified by immunological assay (Western blotting after analysis of total protein by SDS-PAGE) using rabbit polyclonal antibodies raised against PHA-E as the primary antibody, with HRP-conjugated goat anti-rabbit IgG (BioRad) as the secondary antibody. The protein was visualised using ECL detection system (Amersham) as previously described (Gatehouse et al., 1997).

Stability of transgene expression in line 35 was monitored over three successive generations (T1–T3). In all cases plants were grown in controlled environment in rooms with identical conditions (30 °C; 16 h of light).

The final concentration of the PHA-E in the animal diet was also determined by immunoassay and was estimated as 1.5% of the total soluble protein content of the diet.

2.1.4. Southern blot analysis of the PHA-E-expressing rice genome

DNA was extracted from leaf tissue with cetylmethylammonium bromide (CTAB) (Gibb and Padovan, 1994). The aliquots of (10 μg) DNA were digested with appropriate restriction endonucleases (which do not cut the DNA used as the probe, i.e., *pha-e* coding region) and subjected to electrophoresis on 0.8% agarose gels. ^{32}P-labelled hybridisation probes were prepared using the Megaprime random primer labelling kit (Amersham). Transfer of DNA onto nylon membranes, electrophoresis, and hybridisation were all carried out according to standard procedures (Sambrook and Russel, 2001).

2.1.5. Construction and production of recombinant PHA-E lectin

The construct for PHA-E was expressed in a *P. pastoris* strain X33 obtained from Invitrogen (Breda, Netherlands). After optimisation of the fermentation at small-scale (Baumgartner et al., 2002), large-scale fermentations were carried out to produce larger gram quantities. The recombinant PHA-E was purified using a combination of ion-exchange, affinity and hydrophobic-interaction chromatography. Purity was assessed by SDS-PAGE analysis (Baumgartner et al., 2002).

2.2. Compositional analyses of test material

The whole rough rice was dehulled using a testing husker THU 35B (Sakate Corporation, Japan) and milled by a hammer mill, SB-89 (United