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ScienceDirect

Food and Chemical Toxicology 45 (2007) 339–349

www.elsevier.com/locate/foodchemtox

A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats

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Received 14 October 2005; accepted 1 September 2006

Abstract

An animal model for safety assessment of genetically modified foods was tested as part of the SAFOTEST project. In a 90-day feeding study on Wistar rats, the transgenic KMD1 rice expressing Cry1Ab protein was compared to its non-transgenic parental wild type, Xiushui 11. The KMD1 rice contained 15 mg Bt toxin/kg and based on the average feed consumption the daily intake was 0.54 mg Bt toxin/kg body weight.

No adverse effects on animal behaviour or weight gain were observed during the study. Blood samples collected one week prior to sacrifice were analyzed and compared for standard haematological and biochemical parameters. A few parameters were significantly different, but all within the normal reference intervals for rats of this breed and age and not in relation to any other findings, thus not considered treatment related. Upon sacrifice a large number of organs were weighed, macroscopic and histopathological examinations were performed with only minor changes to report.

The aim of the study was to use a known animal model in performance of safety assessment of a GM crop, in this case KMD1 rice. The results show no adverse or toxic effects of KMD1 rice when tested in the design used in this 90-day study. Nevertheless the experiences from this study lead to the overall conclusion that safety assessment for unintended effects of a GM crop cannot be done without additional test group(s).

Keywords: Genetically modified rice; *Bacillus thuringiensis*; Cry1Ab protein; Animal study; Wistar rats; SAFOTEST; Food safety; Feeding trial

1. Introduction

Bt rice is rice that has been genetically modified to express insecticidal genes (*cry* genes) from *Bacillus thuringiensis* (Bt). The transgenic rice is resistant to major lepidopteran insect pests of rice and thus has the potential to significantly decrease yield losses, reduce the use of broad-spectrum chemical insecticides, and furthermore reduce levels of mycotoxins, one of the unexpected benefits of reducing larval attacks (Cheng et al., 1998; Papst et al., 2005). The Bt rice line, KMD1, since its development in 1998 has been characterized thoroughly at the molecular level, and in numerous field trials has shown evidence of

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0278-6915/$ - see front matter
doi:10.1016/j.fct.2006.09.001

affording the farmer a high level of resistance against at least eight different insect pest species (Shu et al., 2000; Ye et al., 2001, 2003).

In spite of the extensive research that has been conducted in developing this pest resistant rice, as summarized by High et al. (2004), Bt rice is not yet grown commercially. Other Bt crops, expressing a range of different *cry* genes, are commercially grown in many parts of the world including Bt corn, Bt cotton, Bt canola and Bt potatoes. Bt toxins (Cry proteins) have been used as microbial pesticides for many years and have a long history of safe use (Mendelsohn et al., 2003; Betz et al., 2000).

Cry proteins show highly species-specific toxicity against certain insects and only a few insect species are affected by each of the Cry proteins. The mode of action in the insect is through specific receptors in the gut, which is highly alkaline, with binding of the toxin resulting in pore-formation, osmotic imbalance, cell lysis and subsequently death of the insect (Betz et al., 2000).

The Cry proteins are regarded harmless or nontoxic to mammals, including humans, probably due to acidified gut pepsinolysis and the lack of Cry protein binding-sites on mammalian gut epithelial cells. Numerous data from toxicity studies show no significant adverse effects of the Cry proteins on body weight gain or clinical observations. Furthermore, no signs of pathogenicity to mammals, including humans, have been reported (McClintock et al., 1995).

Investigations on the effects of the Cry1Ab protein on mammalian cells have revealed no significant effect on bovine hepatocyte morphology or on albumin secretion *in vitro* (Shimada et al., 2003). In animal studies no significant differences were observed in general health or growth rate in pigs fed a Bt corn diet (Chowdhury et al., 2003), although in 1998 Fares and El Sayed observed fine structural microscopic changes in the ileum of mice fed Cry1 potato diet. Bt toxin released by the crop root or from the biomass of Bt corn has been found nontoxic to soil bacteria (Saxena and Stotzky, 2001).

This study is part of the EU-project SAFOTEST, designed to develop scientific methodologies for assessing the safety of genetically modified (GM) crops. The aim of the present 90-day study in Wistar rats was to perform a comparative safety assessment study of the genetically modified Bt rice, KMD1, expressing Cry1Ab in an animal model, when compared to the parental wild-type rice, Xiushui 11, and to furthermore monitor changes in major aerobe and facultative anaerobe bacterial populations in the intestines of the rats.

The study design includes two test groups given comparable diets containing 60% raw brown rice flour from parental and transgenic rice, respectively, to be tested in a directly comparative 90-day feeding study without spiking of the recombinant protein.

The objective was to have identical cohorts of male and female rats in a sub-chronic 90-day exposure to 60% rice diets, which contained realistic and meaningful levels of the transgene-expressed Cry protein, Cry1Ab. The focus was first and foremost on the tissues and organs in initial contact with the diets. These are the digestive tract and related organs, including a detailed veterinarian and pathological assessment of the whole animals' well-being and behaviour.

The rice materials tested in the 90-day toxicity study were subjected to comprehensive analytical characterization before the study so that the compositional data could provide the basis for the interpretation of any possible effects detected in the feeding studies. Every effort was made to provide a consistent and well-characterized GMO diet to the test animals, in a universally adoptable and approvable manner, for a study based on the OECD Guideline no. 408 (OECD, 1995).

2. Materials and methods

2.1. Test material

Bt rice KMD1 and the corresponding parental rice Xiushui 11, were accessed from University of Ottawa (Canada) and Zhejiang University (China), respectively. Seeds of KMD1 and its parental line, Xiushui 11, were produced in the late season of 2000 in Hangzhou, China. Wu et al. (2001) have described generation and selection of the transformant rice. During multiplication of rice seeds, the performance of these materials was consistent with previous years' observations. Neither leaf folders nor stem borers damaged plants of KMD1, while Xiushui 11 was infested by both, leading to curled leaves (caused by leaf folders), dead-hearts and white-heads (by stem borers at vegetative and heading stage, respectively) in the field. All shipping and handling was conducted to protect the freshness and quality of the rice grains. On arrival by air courier at the Danish Institute for Food and Veterinary Research (DFVF, Søborg, Denmark) the rice was stored at 5 °C, before dehulling, grinding and subsequent storage at −18 °C until use. Samples of intact rice grains representative for the bulk material were shipped to Technical University Munich (Germany).

2.2. Characterization of test material

Rice plants were generated by *Agrobacterium*-mediated transformation and positive transformants selected on the basis of hygromycin resistance (Wu et al., 2001). The presence of the Cry1Ab transformation cassette was confirmed by PCR and Southern blot analysis using standard protocols (Sambrook and Russell, 2002). Transgene expression of Cry1Ab in mature seeds of line KMD1 was verified by immunological assay (Western blotting after analysis of total protein by SDS-PAGE) using rabbit polyclonal antibodies raised against Cry1Ab as the primary antibody, with HRP-conjugated goat anti-rabbit IgG (Bio-Rad) as the secondary antibody. The protein was visualized using ECL (chemiluminescence) detection (Amersham) as previously described (Gatehouse et al., 1997) and quantified by densitometric scanning using Bio-Rad Molecular Analyst software. The final concentration of Cry1Ab in the animal diet was also determined by immunoassay.

2.3. Compositional analyses of test material

Intact rice grains were manually dehulled by means of a wooden rice dehuller and ground using a cyclone mill equipped with a 500-μm sieve. The rice flour obtained was immediately frozen and stored at −20 °C until analysis.

Proximates (moisture, starch, fibre, sugars, protein, fat, ash), amino acids, fatty acid distribution and minerals were determined using validated standard protocols (VDLUFA, 1996; VDLUFA, 1997). The content of