World Rabbit Science

World Rabbit Sci. 2013, 21: 257-261
doi:10.4995/wrs.2013.1188

EFFECTS OF TRANSGENIC POPLAR LEAVES WITH BINARY INSECT-RESISTANCE GENES USED AS FEED FOR RABBITS

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Abstract: The aim of this work was to explore the potential toxicological effects on rabbits of transgenic poplar (*Populus cathayana* Rehd.) leaves with binary insect-resistance genes used as feed. Fifty-four 40-d-old weaned New Zealand White Rabbits (*Oryctolagus cuniculus*) (live weight 0.98±0.1 kg) were fed for 70 consecutive days with a common pelleted diet and fresh poplar leaves containing a chitinase-BmkIT gene combination or untransformed counterparts (60 g/d). Rabbit body weight and hematological and biochemical data in blood samples were recorded. Organ histological structures were observed and the organ weights in the 2 groups were also measured. The results of the growth study revealed no significant differences ($P>0.05$) for final mean BW of rabbits, intake of the combined feed and poplar leaves or feed conversion ratio between the 2 groups. No obvious pathological changes were observed in the small intestine, stomach, spleen, kidney, lung, heart, bladder, pancreas, prostate and ovary. Electron microscopic observation of liver cells and renal cells showed they were both normal in the 2 groups. All hematological and biochemical data tested fell within the normal range in the 2 groups after 70 d of feeding. We conclude that the poplar leaves with the chitinase-BmkIT gene combination had no obvious harmful effects on rabbits.

Key Words: growing performance, blood haematology and biochemistry, rabbit, transgenic poplar.

INTRODUCTION

New varieties of crop plants with enhanced features such as protection against common pests, tolerance to herbicides and improved quality traits have been produced by agricultural biotechnology. Notable examples are the genetically modified crops with the "*Bt*" gene that expresses an insecticidal protein (Fujimoto *et al.*, 1993). However, public concern has been voiced about the food safety of these transgenic crops in regard to human consumption and production of animal products (e.g., meat, milk, and eggs) from farm animals fed on transgenic crops. Tudisco *et al.* (2010) reported that plant DNA sequences from low copy number genes of barley (*Hordeum vulgare* L.) grain and soybean (*Glycine max* L. Merr.] meal can be detected in blood, liver, kidney, spleen, muscle tissue and digesta of rabbits. The *Bt* gene is the most effective and widely used insect toxin gene. Various studies have been conducted to evaluate the effect of *Bt* crops on animals (Michelle *et al.*, 2007; Guertler *et al.*, 2010; Grønsberg *et al.*, 2011; McNaughton *et al.*, 2011). Bioassays to determine insect resistance have revealed that the chitinase-BmkIT combination has lethal or growth-inhibiting effects on diamondback moth (*Plutella maculipenis*) larvae (Wang *et al.*, 2005) and fall webworm (*Hyphantria cunea*) larvae (Yang *et al.*, 2008), which confirms that the chitinase-BmkIT combination could be used as a new pest-resistant gene source and might be a complementary alien gene source to the *Bt* toxin gene. It is therefore necessary to assay the effects of chitinase-BmkIT plants on animals. Food processing operations such as thermal preservation, dry-milling or wet-milling often degrade and denature proteins, giving rise to loss of their biological activity (Betz *et al.*, 2000). However, green fodder is a more favoured feed by certain herbivorous mammals. As green forage is directly chewed without thermal processing, the effects of

Correspondence: Y. Sun, sunyi692003@163.com. Received *April 2012* - Accepted *April 2013.*
http://dx.doi.org/10.4995/wrs.2013.1188

transgenic green fodder on animals should be monitored. As a typical pioneer species, poplar grows quickly and its leaves are used as animal feed, which, in composition (%), is: 6.1 crude protein, 6.8 crude fat, 23.9 crude fibre, 16.2 crude ash, 47.0 nitrogen-free extract (Gu *et al.*, 2012). Here we report the evaluation of fresh transgenic poplar leaves harbouring a binary insect-resistant combination (chitinase-BmkIT) fed to rabbits.

The chitinase gene was cloned from *Manduca sexta* (Kramer *et al.*, 1993) and the BmkIT gene encoded a scorpion insect toxin from *Buthus martensii* Karsch (Zhang *et al.*, 2004). Chitinase is an enzyme capable of hydrolysing insoluble chitin to its oligo and monomeric components. The BmkIT gene produces a scorpion neurotoxin of the contractive paralysis type, which causes little or no harm to mammals and acts only on insects (Barton, 1990). The aim of this work was to study the effects of fresh transgenic poplar leaves on growth performance, organ weights, internal tissue histology and hematological and biochemical data of rabbits fed transgenic compared to unmodified poplar leaves.

MATERIALS AND METHODS

Animals and diets

A total of 54 weaned 40-d-old New Zealand White Rabbits (*Oryctolagus cuniculus*) (live weight 0.98±0.1 kg) were provided by the Institute of Animal Husbandry and Veterinary Sciences, Shanxi Academy of Agricultural Sciences (SAAS), China, and randomly assigned into 2 homogeneous groups of 27 each with equivalent males and females. The rabbit diet consisted of a compound feed, described below, supplemented with fresh poplar leaves from transgenic (*Populus cathayana* Rehd.) or non-transgenic control poplar trees. The compound feed was pelleted and consisted of dehydrated alfalfa (*Medicago sativa* L.) (45%), maize (*Zea mays* L.) meal (20%), wheat (*Triticum aestivum* L.) bran (20%), soybean meal (13%), $CaHPO_4$ (1.0%), salt (0.5%), and mineral and vitamin premix (0.5%), which was provided by the Institute of Animal Husbandry and Veterinary Sciences, SAAS.

Experimental procedure

The feeding experiment was conducted for 70 consecutive days. Rabbits were fed individually in cages of 1×1×0.8 m. The environmental conditions (temperature, relative humidity, and air exchange) were controlled (16-29 °C and 40-60% relative humidity). All rabbits in both groups were provided daily with the same amount of compound feed and 60 g/d fresh poplar leaves. To ensure that the poplar leaves were consumed completely, the leaves were provided to rabbits in the early morning and then the compound feed in the afternoon.

Rabbit body weight (BW) was recorded at the beginning and end of the experiment and feed intake was recorded. Feed conversion ratio (FCR) was determined by total feed consumption (g)/weight gain (g), in which fresh-leaf intake and compound feed were expressed in dry weight (28.8 and 89.5% dry matter (DM), respectively). Just before the start of the experiment, a blood sample (3-5 mL) from each rabbit in the 2 groups was obtained from the auricular vein. At the end of the experiment, blood samples were collected again from the same rabbits. Hematological and chemical data were measured by the Provincial Chinese Medicine Hospital, Shanxi, China. At the end of the experimental period (110 d of age), 6 rabbits were dissected from each group and internal organs were observed and weighed in an experimental slaughterhouse without fasting. The thymus, lung, heart, spleen, liver, pancreas, prostate, kidney, bladder, and uterus were removed and their surface liquid was absorbed with filter paper immediately before being weighed. They were fixed in 10% neutral buffered formalin (diluted with phosphate buffer, pH 7.0) after being washed in 0.9% NaCl solution. Strict sanitation protocols were observed during sample collection. Gloves were changed between every sample and table covers were changed as they became soiled. All dissection instruments were sterilised beforehand and rinsed thoroughly with saline to prevent cross contamination. Each sample was dissected with a new sterile scalpel blade.

Sample processing

Six samples of aseptically-taken tissues were embedded in paraffin and sliced into 5 µm thick sections, stained with hematoxylin and eosin (HE) and observed with a light microscope (Zhou *et al.*, 1995). Electron microscopic slices of

liver and kidney were taken by the Medical Experimental Centre of Shanxi Medical University and observed with a transmission electronic microscope (TEM) JEOL JEM-1011(JEOL, Japan), Morada soft imaging system (Kobayashi, 1985).

Statistics analysis

The collected data were expressed as average values and standard errors of the means. Differences between the 2 groups were evaluated using an Independent-Samples T-test, SPSS (12.0) program.

RESULTS AND DISCUSSION

All rabbits in both groups were in good health and lived to the end of the trial. There were no significant differences ($P>0.05$) between control and treatment groups in the final mean BW of rabbits (2196±180 g on average), intake of the compound feed and fresh poplar leaves (on av. 73.66±6.21 g DM/d and 60 g FM/d, respectively) and feed conversion ratio (4.79±0.48 on av.).

After 70 d of feeding, all hematological and biochemical parameters fell within normal ranges in both the treated and control rabbits, and there was no significant difference between the 2 groups (Tables 1 and 2). There were no visibly abnormal symptoms in the rabbit organs of each group. There was no significant difference between the 2 groups ($P>0.05$) in the relative weight (% body weight) of heart (0.26±0.02), liver (2.70±0.01), spleen (0.05±0.01), lung (0.28±0.02), kidney (0.33±0.01), and bladder (0.07±0.01).

In general, no visible pathological changes in tissues were observed between the 2 groups. The tissue morphology of the small intestine, liver, stomach, spleen, kidney, lung, heart, bladder, ovary and prostate from the 2 groups were all normal and there were no visible differences in morphology between the 2 groups. Because histological-pathological examination with HE stain is not a sensitive indicator and possible tiny changes may not be observed with the light microscope, the liver and kidney samples were observed with transmission electronic microscopy. The results showed that both the liver cells and renal cells were normal in the 2 groups, and there were no differences in the organelles and nucleus between the 2 groups.

On one hand, hematological and biochemical components are influenced by the quantity and quality of feed (Akinmutimi, 2004) and are sensitive to toxic elements in feeds, so they are valuable in monitoring feed toxicity (Oyawoye and Ogunkunle, 1998). On the other, the hematological test and analysis of serum constituents provide crucial information for monitoring the health of the animal (Aldrin *et al.*, 1982; Marco *et al.*, 2003). In particular, these data provide reliable information on metabolic disorders, deficiencies and health status before the clinical symptoms become apparent (Kececi *et al.*, 1998; Qiao, *et al.*, 2012). In our study, no significant differences

Table 1: Effects of unmodified or transgenic poplar leaves on hematological data of rabbits after 70 d of feeding.

	Transgenic group	Unmodified group			
Hematological parameter	Mean	Mean	SEM	*P*-value	Normal range
WBC (10^9/L)	9.87	9.70	0.76	0.36	5-13
RBC (10^{12}/L)	5.81	5.66	0.45	0.45	3.8-7.9
HGB (g/L)	115.0	115.0	2.3	0.8	94-174
MCV (fL)	60.9	62.2	1.4	0.4	50-75
MCH (pg)	19.9	20.4	1.4	0.4	18-24
PLT (10^9/L)	549.3	595.7	38.2	0.3	200-650
NE (%)	57.3	34.2	8.7	0.1	34-70
EO (%)	0.60	1.30	0.09	0.14	0-2
BA (%)	0.84	0.80	0.07	0.36	0-0.84
LY (10^9/L)	3.67	4.20	0.25	0.24	3-9
MO (10^9/L)	0.13	0.33	0.08	0.07	<0.5

WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; PLT, platelet count; NE, granulocyte; LY, lymphocyte; MO, monocyte; EO, eosinophil; BA, basophil.

SEM: standard error of the means.

Normal range resource: http://www.medirabbit.com/EN/Hematology/blood_chemistry.htm

Table 2: Effects of unmodified or transgenic poplar leaves on biochemical data of rabbits during a 70-day feeding trial.

	Transgenic group	Unmodified group			
Biochemical data	Mean	Mean	SEM	*P*-value	Normal range
TP (g/L)	52.4	52.8	5.18	0.45	50-75
ALB (g/L)	31.6	31.7	3.25	0.67	25-40
GLO (g/L)	29.8	32.2	3.01	0.25	25-40
GGT (IU/L)	6.17	5.3	0.54	0.44	0-7
ALP (IU/L)	44.4	56.8	5.27	0.28	10-70
AST (IU/L)	30.7	26.7	3.14	0.57	10-98
CHO (mmol/L)	1.37	1.33	0.22	0.19	0.1-2.0
TG (mmol/L)	1.74	1.62	0.26	0.22	1.4-1.76
BUN (mg/L)	17.07	25.33	2.25	0.56	13-30
Creatine (µmol/L)	77.0	86.7	9.27	0.14	53-124
GLU (mmol/L)	6.33	6.41	0.85	0.10	4.2-8.9
Ca (mmol/L)	3.59	3.31	0.43	0.21	3.0-5.0
K (mmol/L)	4.68	4.34	0.51	0.19	4.0-6.5
Na (mmol/L)	152.7	151.0	10.2	0.21	130-155
Cl (mmol/L)	94.3	97.3	10.3	0.26	92-120

TP, total protein; ALB, albumin; GLO, globulin; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; CHO, cholesterol; TG, triglyceride; BUN, blood urea nitrogen; GLU, glucose; Ca, calcium; K, potassium; Na, sodium; Cl, chloride.

SEM: standard error of the means.

Normal range resource: http://www.medirabbit.com/EN/Hematology/blood_chemistry.htm

appeared in the hematological and biochemical parameters between the 2 groups after 70 d of feeding and all the indices fell within normal ranges.

In our study we found no pathological symptoms in samples of small intestine, liver, stomach, spleen, kidney, lung, heart, bladder, prostate and ovary taken from the 2 groups of rabbits. Bone (1979) reported that abnormalities in liver and kidney weights would be observed if there is any toxic element in the feed, due to an increased metabolic rate of the organs in an attempt to reduce these toxic elements or convert anti-nutritional agents to non-toxic metabolites. So, it is common practice in feeding trials to use weights of certain internal organs such as liver and kidney as toxicity indicators. In our study, neither the liver nor kidney weight differed significantly between the 2 groups. Moreover, the histological structures of both the liver and kidney were normal; the observations of liver and renal cells with transmission electronic microscopy further verified the results.

In the present study, we mainly focused on the potential toxicological effects of transgenic poplar leaves on rabbits. It would be important to determine in future trials if transgenic DNA is retained in rabbit tissues and the fate of the corresponding protein. Other aspects such as effects on the development or response of the immune system would also be important.

In conclusion, the present results suggest that insect-resistant transgenic poplar leaves harbouring the chitinase-BmkIT combination had no obvious harmful effects on rabbits.

Acknowledgments: We thank Mr. Keliang Ren and Mrs. Yanping Li of the Institute of Animal Husbandry and Veterinary Sciences, Shanxi Academy of Agricultural Science (SAAS) for their technical assistance in rabbit feeding, and Drs. Hong Xiao and Jianzhong Gao of the Pathology Laboratory, Shanxi Medicine Science University Affiliated No.1 Hospital for their histology instruction. The authors are also grateful to Dr. Kirkham, Department of Agronomy, Kansas State University, USA, for her kind help in polishing the manuscript.

The research was supported by the grants of NSFC (31240081), Major Projects for Transgenic Organism Breeding from Chinese Ministry of Agriculture (2013ZX08003-001), Scientific and Technological Project from Shanxi Science and Technology Department (20110311009) and Shanxi Scholarship Council of the People's Republic of China (2011-061).

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