ALLERGENICITY EFFECTS OF BAR-TRANSGENIC RICE RESISTANT TO HERBICIDE ON PREGNANT MICE

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ABSTRACT

For the purpose of investigating on whether rice transgenic with the herbicide Bar gene is allergic to human beings. 120 Kunming mice were randomly divided into the transgenic group and the control group. Each group was fed with Bar-transgenic rice and the same amount of normal variety of rice for 90 days (for three generations). Among the mothers of each group and each generation with 15 days of gestation: 5 samples were randomly selected. Then, a fully automated blood analyzer was used for analyzing blood physiological indicators; The intestinal mucus immunoglobulin A (sIgA), the serum diamine oxidase (DAO) and the IgE were detected by ELISA. The results showed that there were no significant differences in the blood physiological indexes between the transgenic and the control groups. According to the ELISA, the figures in the three indicators (P>0.05) did not vary greatly. On this basis, we attempted to use SDAP, Farrp and NCBI databases to compare and analyze the allergen sequences of phosphorothionin acetyltransferase (PAT), an expression product of herbicide Bar gene. Also, The PAT enzyme had no homology based on the known allergens in the database, hence, it was not likely to cause sensitization. As a conclusion, under the conditions of this experiment, Bar-transgenic rice had no obvious allergic effects on mice. These results can provide important reference data for risk assessment and promotion of Bar-transgenic rice.

Keywords: *Bar gene, rice, allergenicity, mouse, PAT.*

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Introduction

Rice is one of the most important crops in the world. More than one third of the world's population, among whom Chinese account for 800 million, rely on rice. In recent years, as the annual economic losses caused by weeds account for 10%-20% of the total crop yield, various type of herbicides have been widely developed and used for reducing losses. Among the existing rice germplasm resources, there is rarely any wild rice naturally resistant to herbicides, so conventional breeding is greatly restricted[(1)].

Genetic engineering technology allows the herbicide resistance gene to be introduced for creating new varieties of herbicide-tolerant transgenic rice[(2)]. The herbicide-tolerant transgenic rice provided a new way to prevent weeds, reduce pesticides and protect the environment[(3)].

The herbicide resistance gene is one of the earliest research fields for plant genetic engineering, and has become a success. The success of herbicide-tolerant transgenic rice help remove the chemical weeding problem occurred in the direct seeding of rice[(4)].

By transferring herbicide-tolerant genes into cultivated crops, herbicides can be used more effectively for field weeds, and crops can be protected from phytotoxicity, thereby increasing production and income[(5)]. In 1998, Zhejiang Jinsui Agricultural Genetic Engineering Co., Ltd. cultivated herbicide-resistant transgenic (BAR) rice. In order to evaluate the safety of the transgenic rice, Wang Yin et al. conducted an acute toxicity test, a mutagenic test and a 30-day feeding test in rats respectively[(6-7)]. The results showed that the herbicide-resistant gene (BAR) was not toxic or mutagenic to rice.

In the 30-day feeding test, the rats in each group grew normally, and the indicators from the blood routine were within the normal range[8-10]. Herbicide-tolerant traits had been dominant in all GM crop traits[11], however, there was still a shortage of reports on the effects of bar-transgenic rice on the blood physiological parameters and allergenicity of mice during pregnancy.

In this study, Kunming mice (M. musculus) fed with Bar-transgenic rice for three consecutive generations. Their health and safety were evaluated in terms of hematology, serum biochemistry, sensitization, and sequence comparison of phosphofilament acetyltransferase (PAT) gene. The purpose was to provide reference for the application of Bar-transgenic rice and other transgenic rice. It is of great practical significance to provide reference for the application of Bar-transgenic rice and to eliminate people's psychological panic and misunderstanding about genetically modified food.

Materials, apparatus and methods

Materials and apparatus

• Reconstruction and finishing of mouse breeding houses

• Breeding room, mouse cage, feeding apparatus and other types of equipment.

• Rice: Bar-transgenic herbicide-resistant paddy "Xiangliangyou 681 (Xiang 125S/Bar68-1)" and conventional paddy "Xiangliangyou 68 (Xiang 125S/D68)" (produced by the Institute of Subtropical Agroecology, Chinese Academy of Sciences) were processed into rice respectively.

• Basal feed: In order to meet the needs of nutrients such as protein, the AIN-93G feed formula for rodents introduced by the American Society for Nutrition in 1993 was selected and prepared based on the reference standard.

• Mouse diet: 10% fish meal, 30% base feed, 60% bar-transgenic rice or conventional rice, the specific formula is shown in Table (1). The mice were fed twice a day.

Feed formula/100g	Experimental group	Control group
Rice	60g (Bar68-1)	60g (D68)
Basal feed	30g	30g
Fish meal	10g	10g
Total protein (%)	18.8%	18.8%

Table 1: Diet Formulas of Two Groups of Mice.

• Experimental animals: 120 SPF-grade Kunming mice with the weight range from 18 to 24g (provided by Hunan SJA Laboratory Animal Co., Ltd).

• Automatic blood analyzer, SPSS17.0 statistical software and high speed refrigerated centrifuge (TCL-16A).

• Main reagents: mouse sIgA, DAO and IgE ELISA quantitative assay kit (Wuhan USCN life Company).

Experimental methods

Mouse feeding

120 SPF Kunming mice with the weight ranged from 18~24g were randomly divided into 2 groups. For the transgenic group, 60% of Bar-transgenic rice were added to the diet according to the formula (see Table 1), and the same amount of conventional rice were added to the diet of the control group. The other conditions remained the same and unchanged. The mice were fed twice a day for 90 days, one time in the morning and one time in the evening. The experiment was conducted on three generations.

Blood specimen collection

The mice were fasted for 12 hours before the experiment and were given free access to water. On the 15th day of gestation, 5 pregnant mice in each group were randomly selected for eyeball enucleating, vein dilution, rapid shaking, anticoagulation and immediate determination.

Blood sample determination

A fully automated blood analyzer was used for measuring the following indicators: total white blood cell count (WBC), total red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean red blood cell volume (MCV), mean corpuscular hemoglobin concentration (MCH), average red blood cell hemoglobin concentration (MCHC) and platelet content (PLT).

Detection of intestinal mucus sIgA

On the 15th day of gestation, randomly selected 5 pregnant mice from each generation were dissected from the abdominal cavity. The 7cm of intestinal segments above the ileum of cecum were removed and spread vertically on the filter paper. Then, the blood stains were removed. The intestinal segments were cut and picked up by the surgical scissor. All the content inside the small intestine and mucus were scraped into EP tube and mixed with 1mL phosphate buffer solution and stored in 4 refrigerators.

After the sample was centrifuged at 4°C for 20 min in a 1,500×g low temperature centrifuge, the supernatant liquid was produced and placed in a refrigerator at 4 °C. The procedure should be finished based on the ELISA instructions, and the absorbance reading (A value) of each hole was 450 nm.

Detection of serum DAO and IgE

On the 15th day of gestation, 5 pregnant mice from each generation and each group were randomly selected. The eyeballs of the tested mice were removed and put into the EP tube. After standing static for 1 hour, it was centrifuged at 1000 x g for 20 min at the low temperature of 4°C. The upper serum was taken and stored in a refrigerator at 4°C. The procedure should be finished based on the ELISA instructions, and the absorbance reading (A value) of each hole was 450 nm.

Acquisition of PAT amino acid sequence and comparison of allergen protein databases

The three major databases of SDAP, Farrp and NCBI, which can provide abundant information for allergens, and are powerful and complementary to each other, were used to compare the amino acid sequence of PAT protein with known allergens. The amino acid sequences of PAT can be found on the NCBI website at http://ncbi.nlm.nih.gov, that is, Bar gene X17220. The full-length amino acid sequences of phosphinothricin acetyltransferase (PAT) can be obtained by gene information. Then, the amino acid sequences of PAT were compared in three databases: full sequence alignment, 80 aa reading frame sliding alignment and 6 aa window complete alignment, were used for predicting 3 algorithms in SDAP; full sequence alignment and 80 aa reading frame sliding alignment were used for predicting 2 algorithms in Farrp; and full sequence Blastp analysis was used in NCBI. The default values were searched in the retrieval. Then, E-values, alignment length, and consistency percentage were used for inferring whether PAT was sensitized or potentially sensitized.

Experimental data statistics

The method of the mean plus or minus standard error, which is $\bar{x}\pm SE$, is used for the experimental data. Independent sample t-test was implemented by using SPSS17.0 statistical software. Then, $\alpha=0.05$ was used as the hypothesis test standard to analyze the differences among the statistic of the same generation mice.

Result and analysis

Comparison of blood physiological indexes in parental pregnant mice

Comparison of blood physiological indexes between parental pregnant mice in control group and transgenic group

According to the LDS value, the parental pregnant mice in the control group and transgenic group did not have significant changes in the blood indexes ($P>0.05$) (see Table 2) by comparsion.

Items	Control group	Transgenic group
WBC (10^9/L)	6.39±1.71	8.54±4.64
RBC (10^{12}/L)	7.12±1.89	6.86±1.86
HGB (g/L)	150.2± 24.01	158.4±29.07
HCT (L/L)	23.18±21.08	16.44±11.8
MCV (fL)	42.68±8.17	44.82±6.29
MCH (pg)	83.12±35.91	62.28±38.39
MCHC (pg/g)	2240.2±397.56	1499.0±1108.68
PLT (10^9/L)	1025.6±783.23	992.6±348.48

Table 2: Comparison of blood physiological indexes between parental pregnant mice in control group and transgenic group (LSD value).

Note: 1. The data in the table are mean ± standard deviation; 2. Different letters (a, b) labeled in the same column indicate significant difference ($P<0.05$). Unlabeled or identical letters indicate that the difference is not significant.

The experimental results (Fig.1) showed that the total number of WBC in the parental pregnant mice was higher than that in the control group, and the RBC was lower than that in the control group, but the changes were within the normal range. Therefore, it can be said that there was no significant difference (letters for the same pattern are the same, indicating no significant difference. Different patterns indicate significant differences).

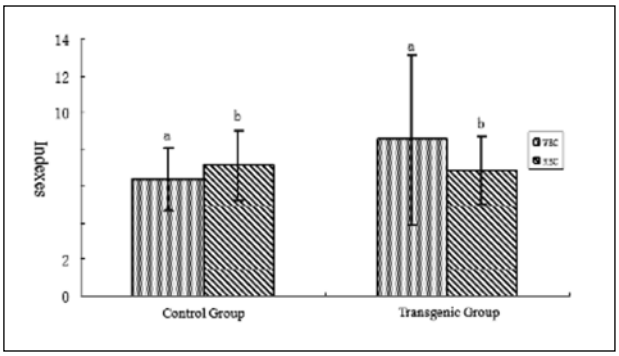

Figure 1: Changes in the number of white blood cells (WBC) and total number of red blood cells (RBC) in the parental control group and the transgenic group.

Note: 1. Letters of the same pattern are the same, indicating no significant difference, and different letters indicate a significant difference. 2. The histogram represents the mean, the H line represents the standard deviation (the same below).

Figure 2 showed that the number of HCT of the parental pregnant mice in the transgenic group was lower than that in the control group, the MCV, higher than that in the control group, and the MCH, lower than that in the control group. However, the numerical changes were within the normal range and there was no significant difference (letters for the same pattern are the same, indicating no significant difference. Different patterns indicate significant differences.)

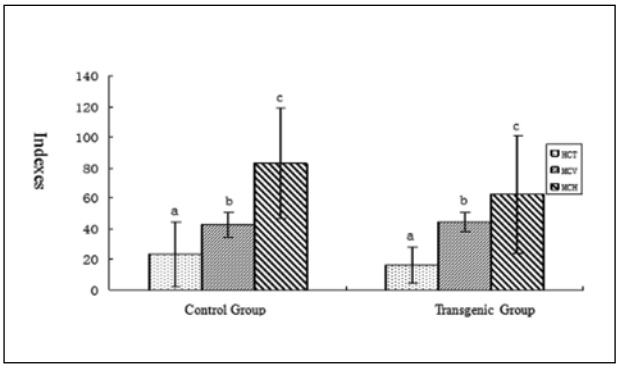

Figure 2: Changes in the values of hematokrit (HCT), MCV and MCH in the parental control group and the transgenic group.

According to the Figure 3, the HGB value of the parental pregnant mice in the transgenic group was slightly higher than that in the control group, the MCHC was lower than that in the control group, and the PLT was also slightly lower than that in the control group.

However, the numerical changes were within the normal range and there was no significant difference (letters for the same pattern are the same, indicating no significant difference. Different patterns indicate significant differences.)

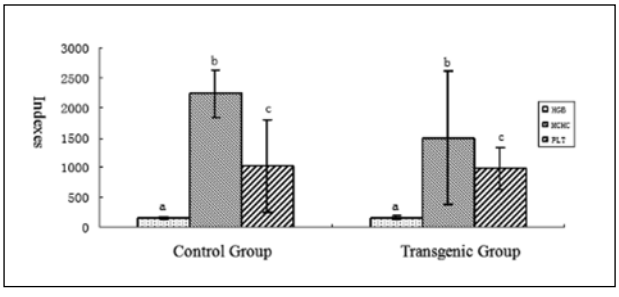

Figure 3: Changes in the values of HGB, MCHC and PLT in the parental control group and the transgenic group.

Comparison of blood physiological indexes of F1-generation pregnant mice

The results of the following Table 3 showed that there was no significant changes of blood indexes of F1-generation pregnant mice between the control group and the transgenic group (P>0.05).

Items	Control group	Transgenic group
WBC (10^9/L)	7.52±2.17	9.92±5.96
RBC (10^{12}/L)	7.45±2.91	4.06±2.78
HGB (g/L)	122.2±47.99	145.6±53.06
HCT (L/L)	29.96±11.28	17.38±12.24
MCV (fL)	43.84±2.13	42.18±3.49
MCH (pg)	24.00±15.86	52.40±32.90
MCHC (pg/g)	515.4±279.6	1293.4±865.2
PLT (10^9/L)	1025.4±525.5	888.4±325.6

Table 3: Comparison of blood physiological indexes of F1-generation pregnant mice between the control group and the transgenic group.

Note: 1 The data in the table are mean ± standard deviation (SD); 2. Different letters (a, b) labeled in the same column indicate significant difference (P<0.05). Unlabeled or identical letters indicate no significant difference.

According to the Figure 4, the WBC of the F1-generation pregnant mice in the transgenic group was higher than that in the control group, but the RBC was lower than that in the control group. However, the numerical changes were within the normal range and there was no significant difference (letters of the same pattern are the same, indicating no significant difference. Different letters indicate a significant difference.)

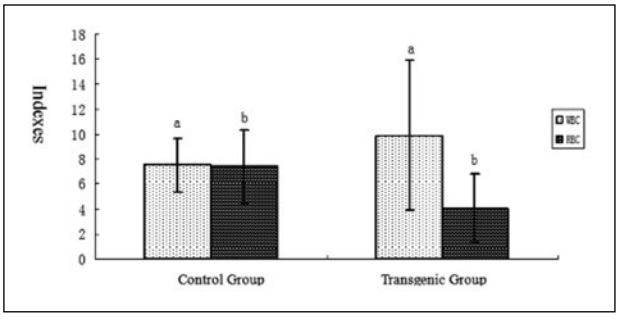

Figure 4: Changes in the values of WBC and RBC between the F1 control group and the transgenic group.

Note: 1. Letters for the same pattern are the same, indicating no significant difference. Different letters indicate a significant difference; 2. The histogram represents the mean number and the H line represents the standard deviation (the same below).

Figure 5 showed that the HCT of the F1-generation pregnant mice in the transgenic group was lower than that in the control group, but the MCV of the F1-generation pregnant mice in the transgenic group was slightly higher than that in the control group. The MCH of the F1-generation pregnant mice in the transgenic group was higher than that in the control group. However, the numerical changes were within the normal range and there was no significant difference (Letters of the same pattern are the same, indicating no significant difference. Different letters indicate a significant difference.)

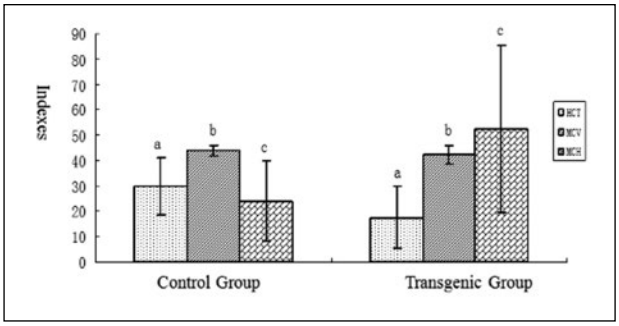

Figure 5: Changes in the values of HCT, MCV and MCH between the control group and the transgenic group.

Figure 6 showed that the HGB of the F1-generation pregnant mice in the transgenic group was slightly higher than that in the control group, but the MCHC of the F1-generation pregnant mice in the transgenic group was slightly lower than that in the control group, and the PLT of the F1-generation pregnant mice in the transgenic group was lower than that in the control group. However, the numerical changes were within the normal range and there was no significant difference (Letters of the same pattern are the same, indicating no significant difference. Different letters indicate a significant difference.)

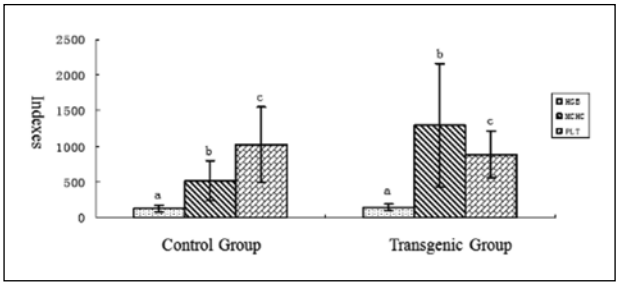

Figure 6: Changes in the values of HGB, MCHC and PLT in the F1 control group and the transgenic group.

Detection of anaphylaxis physical and chemical indicators

Detection of intestinal mucus sIgA

5 pregnant mice were randomly selected from each generation and each group on the 15th day of gestation. The sIgA level was detected by ELISA. The OD value of each sample, or A value, was read at 450 nm by ELISA. The statistical results of SPSS 17.0 are shown in Table 4.

Items	Experimental group	Control group	*P* value between groups
Parental generation	0.703±0.038	0.861±0.065	0.293
F1 generation	0.703±0.019	0.770±0.036	0.180
F2 generation	0.697±0.016	0.701±0.031	0.074

Table 4: Comparison between sIgA statistic in pregnant mice of experimental group and control group (A value, Mean±SE).

Table 4 showed that the absorbance value of sIgA in intestinal mucus of parental pregnant mice (A value) was slightly lower than that of the control group. However, the level of quantitative change was basically within the control group, and there was no significant difference between the two groups (0.703±0.038 vs 0.861±0.065, P=0.293>0.05). Compared with the control group, the content of sIgA in intestinal mucus of F1 and F2-generation pregnant mice had no significant difference (P=0.180, 0.178, 0.078 > 0.05; P=0.074, 0.497, 0.440 > 0.05).

Detection of serum DAO and IgE

The 15th day of gestation witnessed that 5 pregnant mice were randomly selected from each generation and each group. The DAO and IgE levels were detected by ELISA. ELISA was used for OD value of each sample, or A value which was read at 450nm. The statistical results of SPSS 17.0 are shown in Table 5.

Items		Experimental group	Control group	*P* value between groups
Parental generation	DAO	0.884±0.042	0.844±0.061	0.678
	IgE	1.581±0.090	1.313±0.102	0.985
F1 generation	DAO	0.866±0.041	0.815±0.054	0.870
	IgE	1.159±0.054	1.386±0.106	0.078
F2 generation	DAO	0.809±0.040	0.814±0.050	0.497
	IgE	1.609±0.057	1.435±0.090	0.440

Table 5: Comparison of DAO and IgE statistic in pregnant mice of experimental group and control group (A value, Mean±SE).

Table 5 showed that A value of serum DAO in the group of parental pregnant mice with slightly higher median fluctuated slightly violently than that in the control group, but there was no significant difference (0.884±0.042 vs 0.844±0.061, P=0.678>0.05) between both groups. A maximum value of the serum IgE appeared in the experimental group, which was excluded from the analysis. Then, the independent sample t-test between the groups proved no significant difference (1.581±0.090 vs 1.313±0.102, P=0.985>0.05). It was speculated that the maximum value might be caused by errors in the operation or by allergic reactions of the mice themselves. However, considering the multiple reasons behind allergens and the specific charactersitics of IgE, the increasing IgE might be caused by other infections and inflammation, such infected as wound caused by fighting and bites among mice. There was no significant difference in serum DAO content and

serum IgE content between the F1- and F2-generation pregnant mice in both groups (P=0.180, 0.178, 0.078>0.05; P=0.074, 0.497, 0.440 >0.05).

It can be concluded that Bar-transgenic rice did not affect the genetics of mice, and the offspring of the parental experimental group did not become more sensitized after long-term consumption of transgenic rice.

During the 90-day observation period, no pregnant mice in each generation showed obvious allergic symptoms , such as vomiting, diarrhea, and anaphylactic shock.

Acquisition of PAT amino acid sequence and comparison of allergen protein databases

Through the NCBI website at http://ncbi.nlm.nih.gov, the full-length amino acid sequence of the expression product of Bar gene X17220 "phosphinothricin acetyltransferase (PAT)" was obtained through analyzing genetic information, as shown in Figure 7. The full sequence alignment was used in SDAP and the results are shown in Figure 8. Then, the 6aa frame complete alignment was used in the SDAP. The result is shown in Figure 9.

MSPERRPADIRRATEADMPAVCTIVNHYIETSTVNFRTEPQEPQEWTDDLVRLRERYPWLV
AEVDGEVAGIAYAGPWKARNAYDWTAESTVYVSPRHQRTGLGSTLYTHLLKSLEAQGFK
SVVAVIGLPNDPSVRMHEALGYAPRGMLRAAGFKHGNWHDVGFWQLDFSLPVPPRPVLP
VTEI

Figure 7: Full-length amino acid sequence of phosphotinomycin acetyltransferase (PAT).

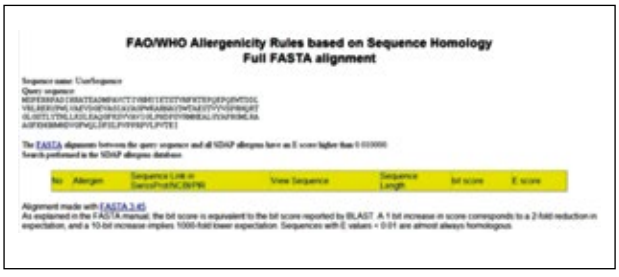

Figure 8: Partial alignment of PAT protein sequence with allergens according to the SDAP database.

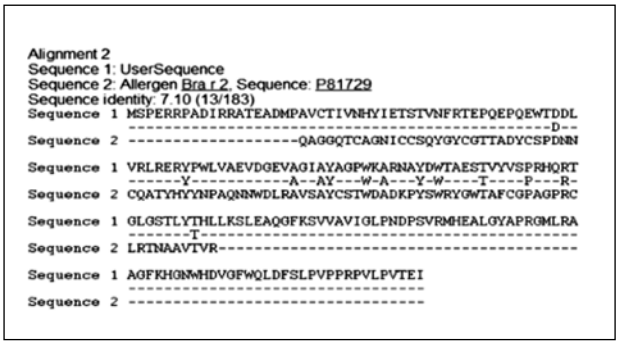

Figure 9: PAT protein sequence alignment search results in the SDAP database.

80 aa reading frame was used in Farrp, and the result is presented in Figure 11 as follows.

Complete sequence alignment was used in Farrp. The results are shown in Figure 10.

Based on the full sequence alignment, 80aa reading frame sliding alignment and 6 aa frame complete alignment in Figure 7-11, it is observed that no similar sequence exists between PAT and known allergens. Therefore, it can be said that the allergenicity of PAT enzyme is very low.

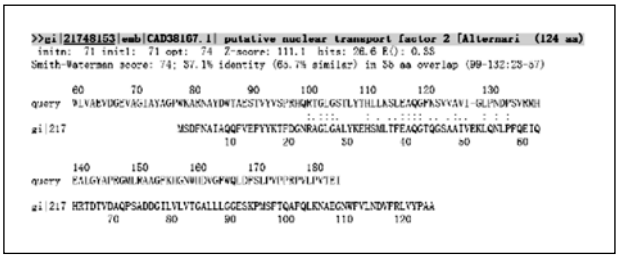

Figure 10: Partial alignment between the PAT protein sequence and the full sequence of the allergen according to the Farrp database.

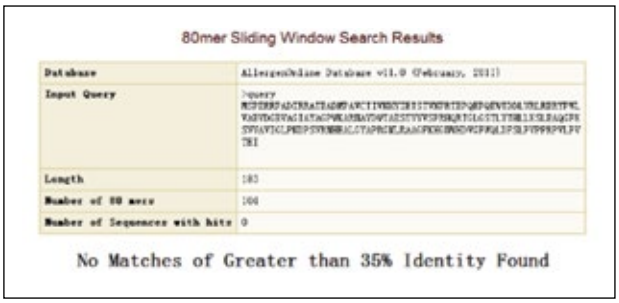

Figure 11: Comparing PAT protein sequence and result of Farrp database 80aa sliding alignment.

Discussion

The routine examination on blood is to judge by observing the change in the number of blood cells and the distribution of the morphology. The three types of cells, namely red blood cells, white blood cells and platelets in the blood, have their own different functions respectively.

In terms of main physiological function, red blood cells serve as a respiratory carrier to meet the breathing needs of the tissue and to enhance the body's motion ability. The significant increase in the number of red blood cells plays an important role in improving the blood circulation of the uterus placenta in pregnant mice, better relieving the metabolic disorder caused by ischemia and hypoxia, and promoting fetal growth. In the parental pregnant mice, the MCV and the HGB of the transgenic group was higher than those of the control group. Compared with the control group, the transgenic group had

slightly lower value in the RBC, HCT and MCH, but all the indicators were within the normal range, and no significant difference occurred. In the F1 generation pregnant mice, the HGB, MCV and MCH values of the pregnant mice in the transgenic group were all slightly higher than those in the control group. However, the MCHC and the PLT was lower than that in the control group, but the numerical changes were within the normal range, and there was no significant difference. Guo Q Y also confirmed that compared with the control group, there were no significant differences in body weight, hematology and serum chemistry results of Male Wistar rats were fed with diets containing BT799 maize[12].

White blood cells can protect the body mechanism and realize immunity through migration, chemotaxis and phagocytosis. Lymphocytosis promotes non-specific immunity in the body. The changes in the number of monocytes and white blood cells affected the body's immune function. The total number of WBC in the parental and F1-generation pregnant mice was more than the control group, but all indicators were within the normal range, which may be related to the biting behavior of the mice.

The main function of platelets is to promote hemostasis and accelerate blood coagulation. At the same time, while platelets also help maintain the integrity of the capillary wall. Studies have shown that during normal pregnancy, the body is in a state of hypercoagulability. This hypercoagulable state is helpful for the physiological self-protection of the maternal mice , especially when the blood coagulation and fibrinolysis are in dynamic equilibrium, which is more conducive to the hemostasis for the placental stripping surface during the delivery process and to the regeneration and repair of the endometrium. The values of platelet content (PLT) in the transgenic and F1-generation pregnant mice were lower than those in the control group. However, the numerical changes were within the normal range, and no significant difference occurred, indicating that Bar-transgenic rice had no significant adverse effect on the reproduction and health of pregnant mice. This is consistent with Liu Qiang's research results[13].

Secretory Immunoglobulin A (sIgA) is the main fluid defense factor for the body in the mucosal immune system. Intestinal mucosal damage and increased permeability of the intestinal wall are the major outcomes for individuals that are allergic to certain type of food. The mucosal immune system with sIgA as the main defense factor contains specific lymphoid tissues, which contact with and phagocytose antigens in the internal environment and induce T lymphocytes and B lymphocyte to react. B lymphocyte-differentiated plasma cells secrete sIgA, which serves as a shelter for maintaining mucosal immunity. If the content of sIgA in the intestinal mucus exceeds the normal level, it means that the intestinal immunity is hyperactive and the food is allergized. Diamine Oxidase (DAO) is a highly active intracellular enzyme which contains deaminated putrescine and histamine. It is a catabolic enzyme for polyamines, such as histamine. More than 95% of DAO is present in mucosal or ciliary epithelial cells of mammalian small intestine. Its activity is closely related to villus height and nucleic acid and protein synthesis of intestinal mucosal cells. When the intestinal mucosal barrier fail, the intestinal mucosal cells fall into the intestinal lumen, and DAO enters the lymphatic vessels and blood veins in the intestinal interstitial space, causing the blood DAO to rise. Therefore, blood DAO activity can reflect intestinal injury and repair. The response in the IgE-mediated immune system is the main side effect of food allergy. The combination of allergen and IgE is crucial to taking the biological activity of allergy into full play. The detection technology for IgE level can be used to test whether the body has allergic reaction.

Based on this experiment, no significant difference occurs in the results of sIgA and DAO test, which shows that the Bar-transgenic rice did not cause damage to the intestinal mucosa and immunity of mice. The impact of genetically modified crops on the gut confirmed that there were no significant difference in most indicators, such as mouse[14], chicken[15], pig[16-17]. This may be due to the fact that the PAT protein is not tolerant to gastrointestinal fluids and will rapidly degrade in the digestive fluids of the organism. There was no significant difference in the detected IgE. No IgE-mediated allergic reaction was found in the three generations of mice fed with Bar-transgenic rice. On the one hand, it could explain the that Bar-transgenic rice can cause no allergic effects, but, on the other hand, this phenomenon might be related to the low expression of Bar gene in rice. Although the tested mice were fed with Bar-transgenic rice for a long time and test on three reproduced generations was also conducted, the trivial amount of PAT protein was far from causing sensitization[18].

Analysis on the amino acid sequence encoded by exogenous genes is the quickest gateway to determine whether food can cause allergic reaction. Generally, allergens are structurally and functionally

conserved, so the homology of the aligned sequences allows for more accurately predicting the alien protein[15,19].

In this thesis, three major international databases for allergen-related information are selected, namely SDAP, Farrp and NCBI, which can provide information, and powerful and complementary for each other, were used to compare the amino acid sequence of PAT protein with known allergens. No similar sequence exists between PAT and known allergens, namely by full sequence alignment, 80aa reading frame sliding alignment and 6aa frame complete alignment, which indicated that PAT enzyme had a very low chance of causing sensitization[20-33].

Conclusions

In this paper, three generations of feeding experiments were carried out with Bar-transgenic rice as experimental material and Kunming mice as model animals. The long-term effects feeding with transgenic rice on the growth and health of the third generation mice were discussed. The results provide data support for the improvement of the food safety evaluation system of GM rice.

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