



Potential allergenicity research of Cry1C protein from genetically modified rice

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ABSTRACT

With the development of genetically modified crops, there has been a growing interest in available approaches to assess the potential allergenicity of novel gene products. We were not sure whether Cry1C could induce allergy. We examined the protein with three other proteins to determine the potential allergenicity of Cry1C protein from genetically modified rice. Female Brown Norway (BN) rats received 0.1 mg peanut agglutinin (PNA), 1 mg potato acid phosphatase (PAP), 1 mg ovalbumin (OVA) or 5 mg purified Cry1C protein dissolved in 1 mL water by daily gavage for 42 days to test potential allergenicity. Ten days after the last gavage, rats were orally challenged with antigens, and physiologic and immunologic responses were studied. In contrast to sensitization with PNA, PAP and OVA Cry1C protein did not induce antigen-specific IgG2a in BN rats. Cytokine expression, serum IgE and histamine levels and the number of eosinophils and mast cells in the blood of Cry1C group rats were comparable to the control group rats, which were treated with water alone. As Cry1C did not show any allergenicity, we make the following conclusion that the protein could be safely used in rice or other plants.

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1. Introduction

Food allergy is an important health issue, it has been estimated to affect 3.7% of the adults in the USA and approximately 6–8% of the children under the age of four, including infants (National Institute of Allergy and Infectious Diseases, 2006; Dioun et al., 2003; Sicherer et al., 2003). An increasing interest in the development of novel foods and, in particular, those derived from genetically modified (GM) crops, has generated considerable debate about the likelihood of adverse health effects including allergenicity. It remains unknown whether the product of a novel gene expressed in a GM crop plant will have the potential to induce *de novo* sensitization or, by cross-reactivity with a known allergen, to elicit hypersensitivity reactions in already-sensitized individuals. Requirements for the safety assessment of the allergenicity of novel foods, and how these requirements might best be addressed

have been widely discussed (Ladics et al., 2010; Taylor and Hefle, 2001; Lack et al., 2002; Helm, 2003; Hollingworth et al., 2003).

One approach is to use the Brown Norway (BN) strain of rats, as this strain is known to be a high immunoglobulin (particularly IgE) responder. Thus, to a certain degree, BN rats resemble atopic humans in their genetic predisposition to react more readily to antigens by producing IgE. BN rats have been used to study oral sensitization to food proteins after administration through the diet or by gavage dosing either in the presence (Atkinson et al., 1996) or absence of an adjuvant (Knippels et al., 1998a,b, 1999a,b, 2000). It has been suggested that, for evaluation of the intrinsic allergenic potential of new proteins, oral application is preferred, and that the presence of an adjuvant is to be avoided. In studies comparing sex differences in BN rats as a model for food allergy, Pilegaard and Madsen (2004) found that females are the sex of choice to be used in experiments.

Oral dosing of peanut agglutinin (PNA) or ovalbumin (OVA) has been routinely used to generate allergic sensitization in BN rats (Ladics et al., 2010), while potato acid phosphatase (PAP) has been used as weak allergenic protein (Dearman and Kimber, 2001). These proteins were used for comparison when determining the potential allergenicity of Cry1C protein.

Bt proteins, derived from *Bacillus thuringiensis*, are insecticidal and have been incorporated into GM crops for pest resistance. However, recent studies have shown that some insects have developed

Abbreviations: BN, Brown Norway; GM, genetically modified; OVA, ovalbumin; PAP, potato acid phosphatase; PBST, phosphate-buffered saline containing 0.02% Tween 20; PNA, peanut agglutinin; TMB, 3,3',5,5'-tetramethylbenzidine.

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resistance to Bt proteins under laboratory and greenhouse conditions (McGaughey, 1985; Hama et al., 1992; Gould et al., 1995; Sayyed et al., 2000; Ferré and Van Rie, 2002; Kain et al., 2004). The Cry1C protein was developed in response to insect resistance to currently available Bt proteins. As Cry1C protein binds a different mid-gut brush border membrane site in the insects than Cry1Aa, Cry1Ab, and Cry1Ac toxins (Alcantara et al., 2004), it can be combined with Cry1A proteins or other groups of Bt proteins to prevent or delay the emergence of pest resistance. The Cry1C toxin is effective against a variety of lepidopteran pests, including rice stem borers. Therefore, Cry1C toxin can be a potential alternative to Cry1A toxins and can also be combined with other Cry1A genes to develop two-toxin Bt crops.

As a new member of the BT insecticidal protein family, the Cry1C protein must be assessed for safety before Cry1C genes can be more widely used in rice or other GM crops. The Cry1C protein was tested for its potential allergenicity in BN rats. Cry1C protein produced in *Escherichia coli* (*E. coli*) was used for the animal studies. As humans contact GM rice orally, we used an oral sensitization protocol without adjuvant to mimic physiological conditions. IgE, IgG2a, cytokine and histamine levels and numbers of blood eosinophils and mast cells were assessed following protein exposure.

2. Material and methods

2.1. Protein production and characterization

The *E. coli* strain PET-30a(+)-Cry1C-*rcp*-BL21 (DE3) (Laboratory of Food Safety, China Agricultural University, Beijing, PR China) was used to highly express Cry1C protein. The Cry1C fusion protein was expressed as previously described by Xu et al. (2009). Purified Cry1C protein was identified by SDS-PAGE, western blot, LC-MS/MS analyses and biological activity tests. The equivalence of *E. coli*- and plant-produced proteins were shown in our previous study (Cao et al., 2010, 2011).

2.2. Chemicals

Peanut agglutinin (PNA), potato acid phosphatase (PAP), ovalbumin (OVA, grade V), bovine serum albumin (BSA) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from local commercial sources. HRP-goat anti-rat IgE and HRP-goat anti-rat IgG2a were purchased from Immunology Consultants Laboratory (USA), Inc. SuperSignal ELISA Femto was purchased from Thermo Fisher Scientific Inc. (USA).

2.3. Animals

All procedures involving experimental animals were performed in accordance with protocols approved by the Committee for Animal Research of Peking University, and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1996). This study was approved by Animal Ethics Committee of the Supervision, Inspection & Testing Center of Genetically Modified Organisms, Ministry of Agriculture, Beijing, and the approval ID of this study is 2009014. Animals were obtained from the Experimental Animal Center of Peking University (Beijing, PR China). Following a 5-day acclimatization period, rats were randomly divided into groups. All animals were kept in stainless steel wire cages with *ad libitum* access to food and water. Animal rooms were maintained at a temperature 21–23 °C with relative humidity ranging from 40–60%; rooms were artificially illuminated (fluorescent lights) with air changes 15 times/hr and a 12 h light/dark cycle.

2.4. Allergenic potential of proteins in BN rats

Five groups of 4-week-old female BN rats ($n = 6$) were used. Rats received 0.1 mg PNA, 1 mg PAP, 1 mg OVA or 5 mg purified Cry1C protein dissolved in 1 mL water by daily gavage for 42 days; adjuvants were not used. Blood samples were obtained from the orbital plexus under light CO₂ anesthesia before sacrifice or by exsanguination from the abdominal aorta at the time of sacrifice. For the detail process of the study please go to Table 1.

2.5. Assays for antigen-specific IgG2a and IgE

For the detection of protein-specific IgG2a, 96-well microtiter plates were coated overnight at 4 °C with 100 µL/well of a 10 µg/mL solution of PNA, PAP, OVA or with a 100 µg/mL solution of Cry1C protein in 0.05 M carbonate buffer, pH 9.6. Plates were washed three times with PBST (phosphate-buffered saline containing 0.02% Tween 20). Next, 100 µL/well PBST containing 1% BSA (PBST/BSA) was added to each well and plates were incubated for 1 h at 37 °C. Plates were then washed and serial dilutions of rat serum in PBST/BSA were added to the wells and incubated for 1 h at 37 °C. After washing, 100 µL/well horseradish peroxidase conjugated goat anti-rat IgG2a diluted 1:5000 in PBST/BSA was added and plates were incubated for 1 h at 37 °C. Plates were then washed again and the enzyme substrate solution TMB (6 mg/mL dimethyl sulfoxide) was added. Plates were developed at room temperature for 5–15 min. Finally, 100 µL/well of 2 M H₂SO₄ was added. Optical densities were read spectrophotometrically at 450 nm with an ELISA plate reader (Thermo Fisher Scientific Inc.). Pooled control serum pool was used as negative control; it was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value to determine the antibody titer. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All samples were run in duplicate. Positive and negative control samples were incorporated for each 96-well plate (Knippels et al., 1998b).

ELISA techniques were used to measure antigen-specific serum IgE antibodies. First, 96-well microtiter plates were coated at 4 °C with a 10 µg/mL solution of PNA, PAP, OVA or with a 100 µg/mL solution of Cry1C protein in 0.05 M carbonate buffer, pH 9.6 for 48 h. Plates were washed three times with PBST. Next, 100 µL/well of PBST/BSA was added to each well and plates were incubated for 1 h at 37 °C. Plates were then washed, and 1:2 dilutions of rat serum in PBST/BSA were added to the wells and incubated for 24 h at 4 °C. After washing, 100 µL/well of HRP-goat anti-rat IgE serum diluted 1:5000 with PBST/BSA was added and plates were incubated at 4 °C for 16 h. Next, plates were washed six times, and 100 µL/well of SuperSignal ELISA Femto was added. Plates were developed at room temperature for 1 min; chemoluminescence was read by TECAN GENios (TECAN Ausreia GmbH).

In this study the titre of the test proteins was determined as follows: A water control group sera pool was used as negative control.

Table 1
The design of experiments.

Days	Process
1–42	Gavage every day
14	Detection for IgG2a, IgE, blood eosinophil and cytokine
28	Detection for IgG2a, IgE, blood eosinophil and cytokine
42	Detection for IgG2a, IgE, blood eosinophil and cytokine
52	Challenge with proteins then detection for histamine at 0, 0.5, 2 1.5 h
52	Challenge with proteins then collection of jejunum and ear tissues

The pooled control group sera was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titre in the test sera. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titre. All analyses were performed in triplicate.

2.6. Cytokine and histamine ELISAs

IFN- γ , IL-4 and Histamine serum concentrations were assessed using RapidBio ELISA Kits (RapidBio.org, USA); all tests were performed according to the manufacturer's instructions.

2.7. Real-time RT-PCR for cytokines

Total RNA was isolated using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany), and cDNA was transcribed with an iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA). Gene expression was determined by means of PCR with an ABI 7500 Thermal cycler (Applied Biosystems, Foster City, CA, USA) and specific Taqman probes (Applied Biosystems) for each gene of interest. β -Actin was used as a housekeeping gene for analysis of changes in cycle threshold values. The fold induction above SEB alone was determined based on changes in the Δ cycle threshold values.

2.8. Challenge effects

Animals previously sensitized with PNA, PAP, OVA and Cry1C were orally challenged 10 days after the final oral gavage of the oral sensitization period. Animals sensitized with protein and control animals were orally challenged with 2 mL of a 5 mg/mL protein solution suspended in tap water. Blood samples were collected from the orbital plexus under light CO₂ anesthesia at 0, 0.5, 1, and 1.5 h after protein administration. Sera were prepared and used for histamine quantification by ELISA (Knippels et al., 1999a).

2.9. Blood eosinophil quantification

Blood was collected into EDTA-coated tubes, and absolute eosinophil numbers were determined using Hemavet 950 FS (Drew Scientific Inc., USA).

2.10. Histology

Jejunum and ear tissues were collected, fixed in formalin, and then embedded in paraffin; tissue sections were then stained with hematoxylin and eosin or toluidine blue for the identification of mast cells. Mast cell numbers and activation status were determined by counting cells with dense metachromatic granules and compact shape versus those with dispersed granules extending clearly outside the cell body.

2.11. Statistical analysis

Statistical comparisons were designed to determine whether the differences in the aforementioned response variables between groups were attributable to the proteins as compared to water group. Data obtained from the PNA, OVA, PAP and Cry1C protein groups were compared separately using the values from the water group. Homogeneity of variance was analyzed by one-way analysis of variance (ANOVA) with the statistical software program Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p < 0.05$, and a step-down analysis was conducted using least squares differences (LSD).

3. Results

3.1. Protein-specific IgG2a and IgE

We investigated antigen-specific immunoglobulin production after weekly administration of PNA, PAP, OVA and Cry1C proteins and compared elicited responses to rats treated with water alone. PNA and OVA promoted a gradual increase in PNA-specific IgE and IgG2a levels in BN rats over time (Fig. 1). PAP induced lower levels of IgG2a or IgE at a later time point compared to PNA and OVA. No Cry1C-specific IgE or IgG2a antibodies were detected in the Cry1C group rats.

3.2. Eosinophils in the blood and jejunum

The number of eosinophils in the water group were comparable with the number in other groups at the 14 days time point; then at the 28 days time point, eosinophil numbers in the PNA and OVA group were higher than the water group. At the last time point (day 42) the number of eosinophils in the PNA group was higher than the OVA group rats (Fig. 2). Histology showed that many eosinophils were present in intestinal villi clearance and intestinal glands clearance in PNA, PAP and OVA groups compared to the water group. However, rats in the Cry1C group exhibited no abnormal changes compared to the water group.

3.3. Histamine in the serum

Histamine production induced by PAN sensitization increased very quickly (within 1 min) so that at the 0 h time point we detected the highest concentration of histamine in the PNA group (Fig. 3); this reading was significantly higher than that in the control group. OVA-induced histamine levels increased slowly and steadily, with the highest concentrations of histamine measured at the 0.5 h time point; histamine levels were sustained for 0.5 h, and then dropped to levels not significantly different compared to rats in the water group. In the PNA group, histamine levels decreased quickly to levels lower than those in rats in the water group at the 1.5 h time point. Histamine concentrations did not increase following antigen challenge in rats from either the PAP or Cry1C groups compared to the water group; levels were not significantly different from the water group at any time point. Regardless of group, all histamine levels returned to normal 1.5 h after the protein challenge.

3.4. Th1 and Th2 cytokines

Fig. 4 illustrates that IL-4, IL-5, IL-10 and IL-13 mRNA expression was significantly enhanced in jejunum tissue from rats sensitized with PNA compared to rats in the water group; cytokine mRNA expression was not significantly different in PAP, OVA and Cry1C groups when compared to the water group except IL-4 and IL-10. However, the production of IFN- γ mRNA was decreased in jejunum tissue from rats in the PNA or OVA group. Reduction in IFN- γ mRNA levels in response to PAP and Cry1C stimulation was relatively modest compared to the water group. Fig. 5 represents that mRNA expression of the Th2-associated cytokines IL-5, IL-10 and IL-13 was increased in spleens from PNA or OVA-treated rats. Conversely, the expression of the Th1-associated cytokine IFN- γ and IL-2 was significantly reduced in the PNA group, and expression of IL-2 mRNA was significantly reduced in the OVA group (Fig. 5). These results were consistent with data generated from cytokine ELISAs of serum samples (Fig. 6).

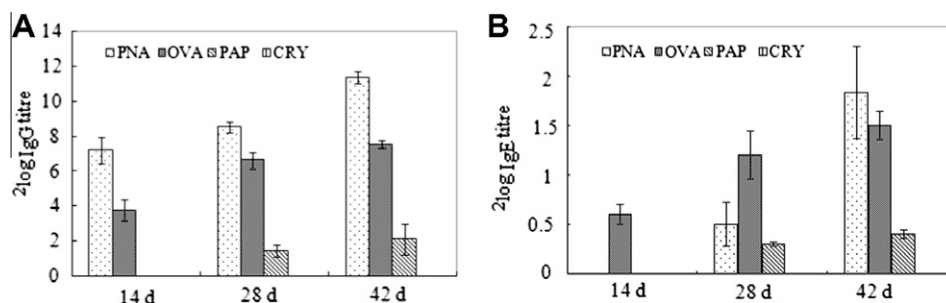


Fig. 1. BN rats were exposed orally to 1 mL of PNA (0.1 mg), PAP (1 mg), OVA (1 mg) or Cry1C (5 mg) daily for 42 days. Protein-specific IgG2a (A) and IgE titers (B) at 14, 28 and 42 days time points were determined by ELISA. Each value represents the mean $\pm$ SD for six mice.

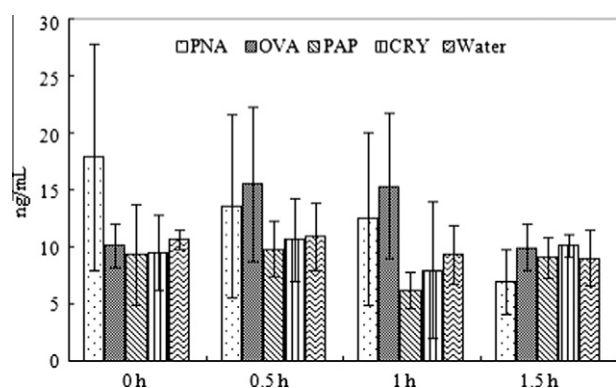


Fig. 2. BN rats were exposed orally to 1 mL of PNA (0.1 mg), PAP (1 mg), OVA (1 mg), or Cry1C (5 mg) daily for 42 days. Blood eosinophil numbers were determined on days 14, 28, and 42 by Hemavet 950 FS. Each value represents the mean $\pm$ SD for six mice.

3.5. Histology

Hematoxylin and eosin staining revealed that in the OVA and PNA groups, neutrophils and goblet cells increased in intestinal

villi clearance and intestinal glands clearance (Fig. 7). There were also some neutrophils and goblet cells in intestinal villi and intestinal glands in PAP and Cry1C groups compared to the water group. Examination of ear tissue revealed that one side of the ear tissues exhibited significant thickening with loose subcutaneous tissue the PNA, PAP and OVA groups. Additionally, some of the rats in the PNA group showed capillary hyperemia. Dyed red crumb material within the dermis was found in the OVA and PNA groups. This might be explained by muscle contraction and increased acid in the muscle in the dermis.

Toluidine blue stains showed increased numbers of mast cells in jejunum and ear tissue sections from the PNA or OVA group. However, only a slight increase in mast cell number was noted in the PAP or Cry1C groups compared to the control group.

4. Discussion

In the previous study we found that the Cry1C protein had no sequence homology with any known allergens by bioinformatics analysis and it was rapidly digested in SGF and SIF (Cao et al., 2010). In addition to the approaches used earlier, there is a need for methods to identify novel proteins with inherent sensitizing

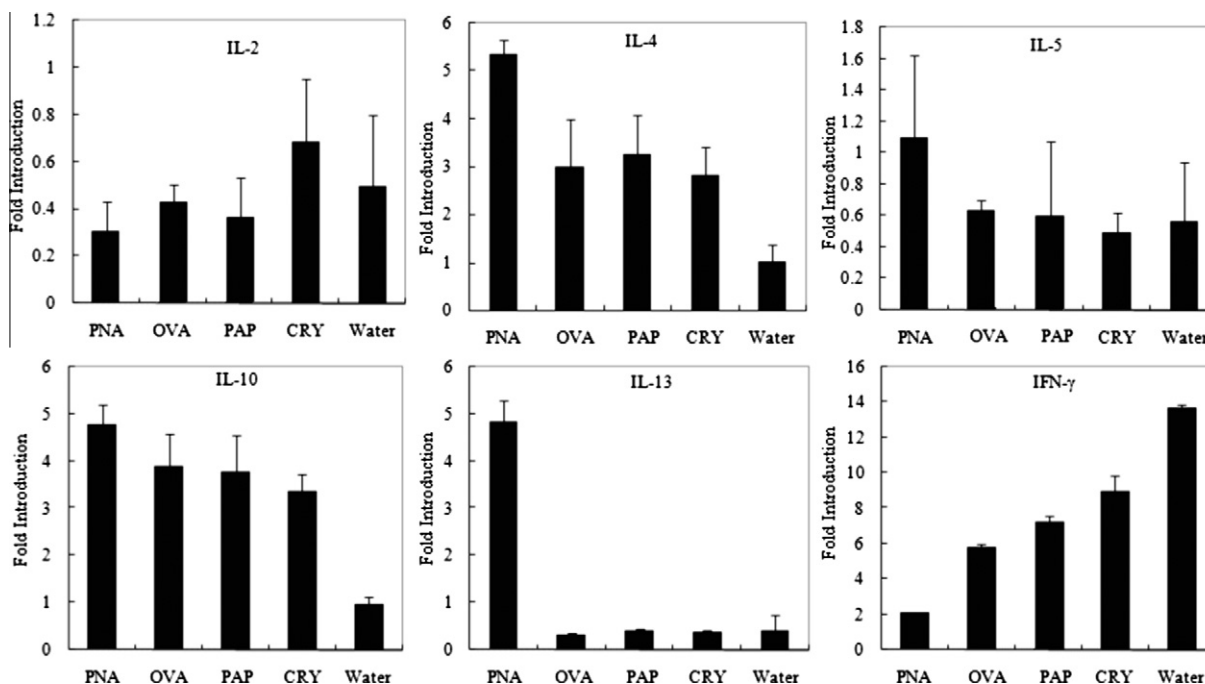


Fig. 3. BN rats were exposed orally to 1 mL of PNA (0.1 mg), PAP (1 mg), OVA (1 mg), or Cry1C (5 mg) daily for 42 days. Ten days after the last gavage, rats were orally challenged with antigens. Antigen-driven histamine levels in the serum of protein-sensitized rats at 0, 0.5, 1 and 1.5 h after antigens challenge were determined by ELISA. Each value represents the mean $\pm$ SD for six mice.

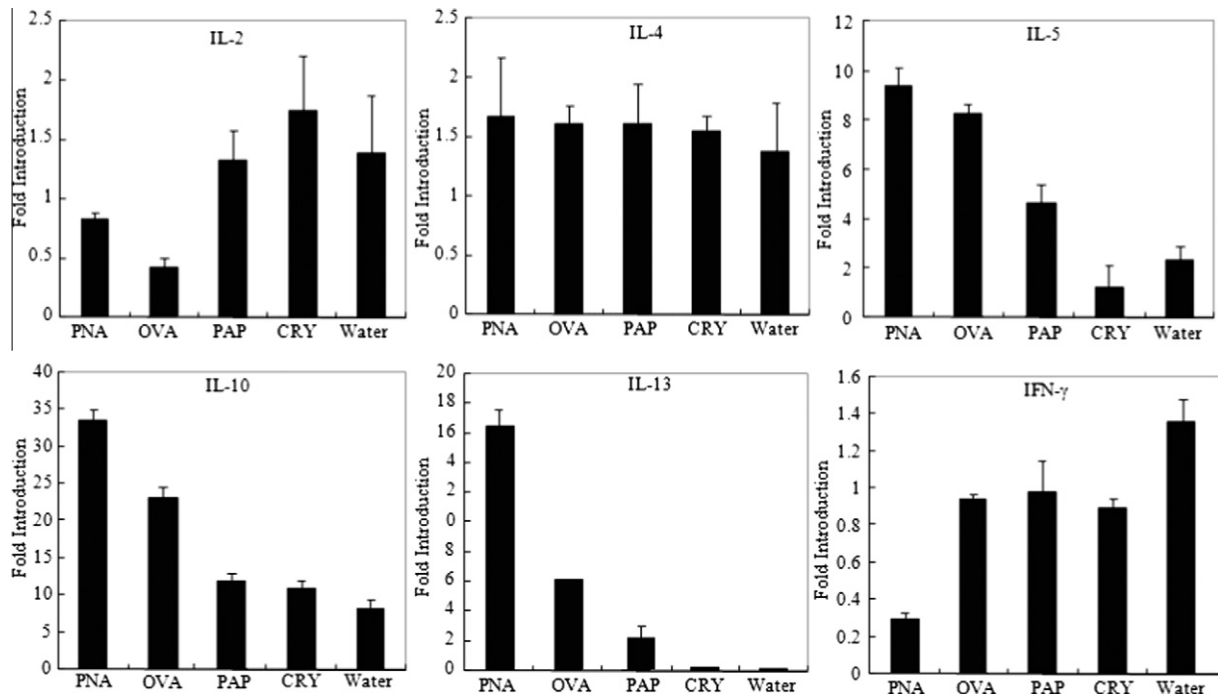


Fig. 4. BN rats received 0.1 mg PNA, 1 mg PAP, 1 mg OVA or 5 mg purified Cry1C protein dissolved in 1 mL water by daily gavage for 42 days. Ten days after the last gavage, rats were orally challenged with antigens. Following euthanasia, jejunum tissue samples were collected and total RNA was isolated. Samples were analyzed for mRNA expression of IL-2, IL-4, IL-5, IL-10, IL-13 and IFN- γ using RT-PCR. Each value represents the mean $\pm$ SD for six mice.

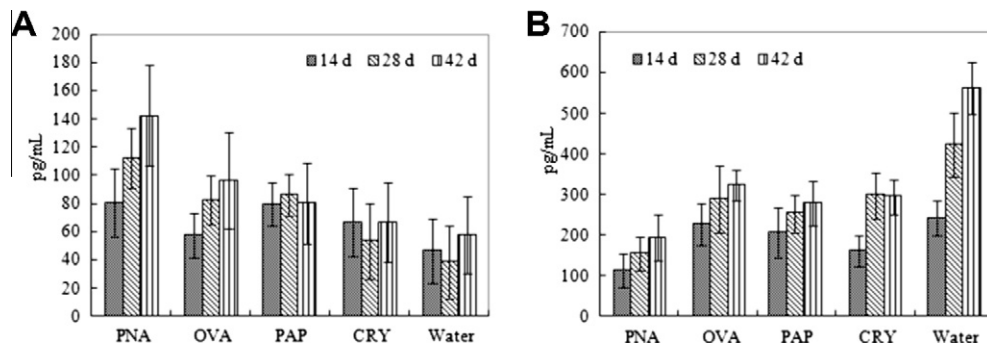


Fig. 5. BN rats received 0.1 mg PNA, 1 mg PAP, 1 mg OVA or 5 mg purified Cry1C protein dissolved in 1 mL water by daily gavage for 42 days. Ten days after the last gavage, rats were orally challenged with antigens. Following euthanasia, spleens were collected and total RNA was isolated. Samples were analyzed for mRNA expression of IL-2, IL-4, IL-5, IL-10, IL-13 and IFN- γ using RT-PCR. Each value represents the mean $\pm$ SD for six mice.

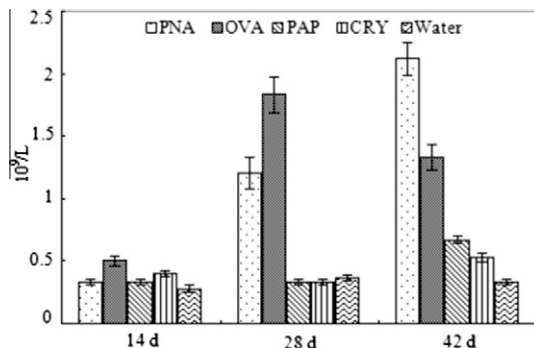


Fig. 6. BN rats received 0.1 mg PNA, 1 mg PAP, 1 mg OVA or 5 mg purified Cry1C protein dissolved in 1 mL water by daily gavage for 42 days. Ten days after the last gavage, rats were orally challenged with antigen. Rats were euthanized and blood were collected. The expression of IL-4 (A) and IFN- γ (B) in serum was detected by ELISA on days 14, 28 and 42. Each value represents the mean $\pm$ SD for six mice.

potential that may lack structural homology (Selgrade et al., 2003) or pepsin-sensitive (Fu et al., 2002). This need was recognized by the consultation panel convened in 2001 by the FAO and WHO; also, they concluded that animal models may contribute valuable information on the likely allergenicity of foods derived from GM crops (FAO/WHO, 2001).

An oral sensitization protocol was developed using BN rats, which avoided the use of adjuvants by presenting multiple doses of sensitizing antigen over a 42 days period (Knippels and Penninks, 2003, 2005; Penninks and Knippels, 2001). Knippels et al. (1998a) reported that daily intragastric administration of 1 mg OVA for 42 days induced both OVA-specific IgG as well as OVA-specific IgE responses as measured by both ELISA and homologous passive cutaneous anaphylaxis assay. IgE responses of rats orally exposed to PNA or OVA increased with time; however, IgE was not detected in the Cry1C or water groups. PAP could cause low levels of the IgE or IgG2a growth. In allergenicity studies, PAP can be used as a weak sensitization protein to provide a point of

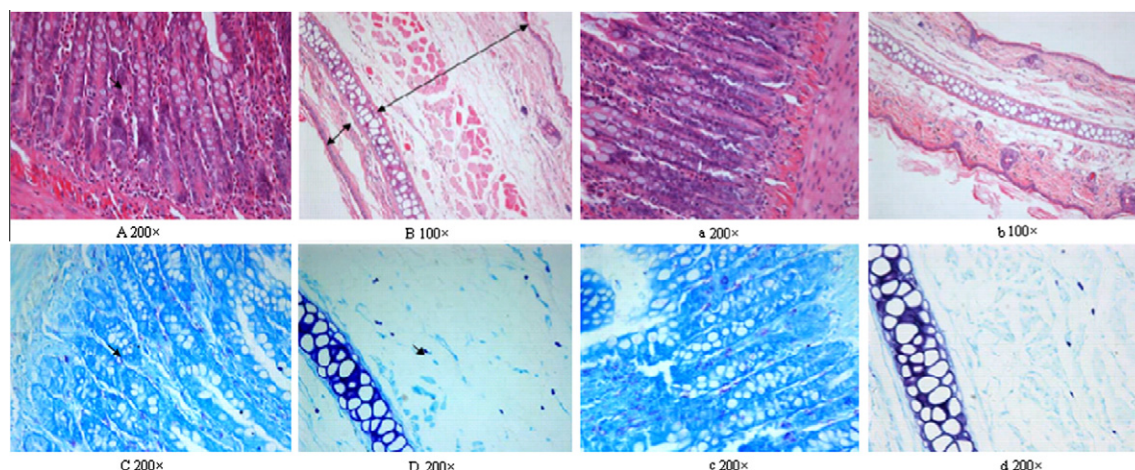


Fig. 7. BN rats received 0.1 mg PNA, 1 mg PAP, 1 mg OVA or 5 mg purified Cry1C protein dissolved in 1 mL water by daily gavage for 42 days. Ten days after the last gavage, rats were orally challenged with antigens. Following euthanasia, jejunum and ear tissues were collected and fixed in formalin. Hematoxylin and eosin, toluidine blue staining of jejunum and ear tissues of PNA-sensitized rats (A, B, C, D) and water group rats (a, b, c, d). (A) Representative histology of tissue stained with hematoxylin and eosin demonstrating a robust eosinophilic infiltration into the jejunum tissues. Black arrows indicate eosinophils. (B) Hematoxylin and eosin staining of ear tissue, illustrating one side of the ear tissues exhibited significant thickening with loose subcutaneous tissue of the PNA group rats. Representative sections showing toluidine blue staining on jejunum (C) and ear (D) tissue from a PNA group rat after challenge. Mast cells were stained with a deep blue color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

comparison for the allergenicity of a new protein (Dearman and Kimber, 2001).

The association between eosinophils and allergic disease has been known for many years. It has been reported that eosinophils may augment and sustain the gastrointestinal inflammatory response through the release of inflammatory mediators and/or granule cationic proteins that are toxic to the mucosa (Sampson, 1999; Dvorak et al., 1993; Kato et al., 1998). Hogan et al. (2000) found allergen can induce accumulation of gastrointestinal eosinophils, which are coincident with our results: both of the blood eosinophils and histology indicated that eosinophils increased in PNA or OVA-treated rats, the increases were statistically significant compared to control group rats.

The histamine in the PNA group rats instantaneously rose to maximum levels, indicating that PNA is a strong allergen. Rats sensitized to OVA, secreted histamine slower but maintained production levels for 0.5 h. The parameters of histamine production and increased numbers of mast cells were used to define authenticated food allergy. As Cry1C protein sensitization did not result in increased histamine levels or increased mast cell numbers, these data suggest that Cry1C is not a allergenic protein.

In this study PNA stimulated the theoretical changes of cytokines. The change trends of cytokines were related to the detection time after challenge (Ferreira, 2003), and the sensitization mechanisms induced by different allergens were not the same (Maciorkowska et al., 2005), which resulted in the cytokine changes different. Cytokine levels also had relations with test materials (Ferreira, 2003). The detection time of cytokines, suitable positive protein for oral sensitization and the corresponding cytokines for different test materials are still need to continue to be researched in the future.

This study proclaims that antibodies, eosinophils and histamine detection can be very sensitive indicators of allergens and non-allergenic proteins. As the system of the food which people orally intake is very complex, some factors may enhance the allergenicity of certain allergens. The genetically modified rice was identified substantial equivalence with the traditional control, except for the inserted protein (the Cry1C protein) (Cao et al., 2012). As there are no reports said the ingredients in rice can result in increased sensitization so the chance of the

ingredients in rice which can increase the allergenicity of the Cry1C protein is very small.

5. Conclusion

In conclusion, BN rats can be used as an animal model for the study of food allergy. The strong allergen PNA or OVA induced antigen-specific IgG2a and IgE. The weak allergen PAP induced lower and later antigen-specific IgG2a and IgE. Cry1C protein-sensitized rats had no antigen-specific IgG2a or IgE. In PNA-treated rats, IgE-mediated allergy was indicated by increased histamine levels immediately following PNA stimulation and increased numbers of mast cells. Additionally, PNA or OVA-sensitized rats had eosinophils in the intestinal villi clearance and intestinal glands clearance. In contrast to the other protein groups tested, the Cry1C group showed no allergy symptoms. Thus, we conclude that Cry1C does not appear to be more allergenic than PAP, and that it is safe to use in food or animal feed. This result is consistent with our earlier conclusions (Cao et al., 2010). In this study, we used several ways to determine the allergenicity of a new protein. These methods could be verified with each other. Additionally, various proteins known to have different allergenic properties were employed to judge the strength of Cry1C protein sensitization. This technique provides a new methodology to assess GM food allergenicity.

6. Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

- Alcantara, E.P., Aguda, R.M., Curtiss, A., Dean, D.H., Cohen, M.B., 2004. *Bacillus thuringiensis* d-endotoxin binding to brush border membrane vesicles of rice stem borers. Arch. Ins. B. 55, 169–177.
- Atkinson, H.A., Johnson, I.T., Gee, J.M., Grigoriadou, F., Miller, K., 1996. Brown Norway rat model of food allergy: effect of plant components on the development of oral sensitization. Food Chem. Toxicol. 34, 27–32.
- Cao, S.S., He, X.Y., Xu, W.T., Ran, W.J., Liang, L.X., Luo, Y.B., Yuan, Y.F., Zhang, N., Zhou, X., Huang, K.L., 2010. Safety assessment of Cry1C protein from genetically modified rice according to the national standards of PR China for a new food resource. Regul. Toxicol. Pharm. 58, 474–481.
- Cao, S.S., Xu, W.T., Ran, W.J., Liang, L.X., He, X.Y., Luo, Y.B., Yuan, Y.F., Huang, K.L., 2011. Expression, purification and refolding of recombinant Cry1C gene obtained in *E.coli* as inclusion bodies. Food Sci. 32, 211–215.
- Cao, S.S., He, X.Y., Xu, W.T., Luo, Y.B., Yuan, Y.F., Liu, P.F., Cao, B., Shi, H., Huang, K.L., 2012. Safety assessment of transgenic *Bacillus thuringiensis* rice T1c-19 in Sprague-Dawley rats from metabonomics and bacterial profile perspectives. IUBMB Life 64 (3), 242–250.
- Dearman, R.J., Kimber, I., 2001. Determination of protein allergenicity: studies in mice. Toxicol. Lett. 120, 181–186.
- Dioun, A.F., Harris, S.K., Hibberd, P.L., 2003. Is maternal age at delivery related to childhood food allergy? Pediatr. A 14, 307–311.
- Dvorak, A.M., Onderdonk, A.B., McLeod, R.S., Monahan-Earley, R.A., Antoniolli, D.A., Cullen, J., Blair, J.E., Cisneros, R., Letourneau, L., Morgan, E., et al., 1993. Ultrastructural identification of exocytosis of granules from human gut eosinophils in vivo. Int. Arch. Allergy Immunol. 102 (1), 33–45.
- Ferré, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 47, 501–533.
- Ferreira, M.A., 2003. Cytokine expression in allergic inflammation: systematic review of in vivo challenge studies. Mediat. Inflamm. 12 (5), 259–267.
- Food and Agriculture Organization/World Health Organization, 2001. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology.
- Fu, T.J., Abbott, U., Hatzos, C., 2002. Digestibility of food allergens and non-allergenic proteins in simulated gastric and intestinal fluids – a comparative study. J. Agr. Food Chem. 50, 7154–7160.
- Gould, F., Anderson, A., Reynolds, A., Bumgarner, L., Moar, W., 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88, 1545–1559.
- Hama, H., Suzuki, K., Tanaka, H., 1992. Inheritance and stability of resistance to *Bacillus thuringiensis* formulations of the diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae). Appl. Entomol. Zool. 27, 355–362.
- Helm, R.M., 2003. Food biotechnology: is this good or bad? Implications to allergic diseases. Ann. Allergy A 90, 90–98.
- Hogan, S.P., Mishra, A., Brandt, E.B., Foster, P.S., Rothenberg, M.E., 2000. A critical role for eotaxin in experimental oral antigen-induced eosinophilic gastrointestinal allergy. Proc. Natl. Acad. Sci. USA. 97 (12), 6681–6686.
- Hollingworth, R.M., Bjeldanes, L.F., Bolger, M., Kimber, I., Meade, B.J., Taylor, S.L., Wallace, K.B., Society of Toxicology ad hoc Working Group., 2003. The Safety of Genetically Modified Foods Produced through Biotechnology. Toxicol. Sci. 71, 2–8.
- Kain, W.C., Zhao, J.Z., Janmaat, A.F., Myers, J., Shelton, A.M., Wang, P., 2004. Inheritance of resistance to *Bacillus thuringiensis* Cry1Ac toxin in a greenhouse-derived strain of cabbage looper (Lepidoptera: Noctuidae). J. Econ. Entomol. 97, 2073–2078.
- Kato, M., Kephart, G.M., Talley, N.J., Wagner, J.M., Sarr, M.G., Bonno, M., McGovern, T.W., Gleich, G.J., 1998. Eosinophil infiltration and degranulation in normal human tissue. Anat. Rec. 252 (3), 418–425.
- Knippels, L.M., Penninks, A.H., 2003. Assessment of the allergic potential of food protein extracts and proteins on oral application using the Brown Norway rat model. Environ. Health Persp. 111, 233–238.
- Knippels, L.M., Penninks, A.H., 2005. Recent advances using rodent models for predicting human allergenicity. Toxicol. Appl. Pharm. 207, 157–160.
- Knippels, L.M., Penninks, A.H., Houben, G.F., 1998a. Continued expression of anti-soy protein antibodies in rats bred on a soy protein-free diet for one generation: the importance of dietary control in oral sensitization research. J. Allergy Clin. Immunol. 101, 815–820.
- Knippels, L.M., Penninks, A.H., Spanhaak, S., Houben, G.F., 1998b. Oral sensitization to food proteins: a Brown Norway rat model. Clin. Exp. Allergy 28, 368–375.
- Knippels, L.M., Penninks, A.H., Smit, J.J., Houben, G.F., 1999a. Immune-mediated effects upon oral challenge of ovalbumin-sensitized Brown Norway rats: further characterization of a rat food allergy model. Toxicol. Appl. Pharm. 156, 161–169.
- Knippels, L.M., Penninks, A.H., van Meeteren, M., Houben, G.F., 1999b. Humoral and cellular immune responses in different rat strains on oral exposure to ovalbumin. Food Chem. Toxicol. 37, 881–888.
- Knippels, L.M., van der Kleij, H.P., Koppelman, S.J., Houben, G.F., Penninks, A.H., 2000. Comparison of antibody responses to hen's egg and cow's milk proteins in orally sensitized rats and food-allergic patients. Allergy 55, 251–258.
- Lack, G., Chapman, M., Kalsheker, N., King, V., Robinson, C., Venables, K., 2002. Report on the potential allergenicity of genetically modified organisms and their products. Clin. Exp. Allergy 32, 1131–1143.
- Ladics, G.S., Knippels, L.M., Penninks, A.H., Bannon, G.A., Goodman, R.E., Herouet-Guicheney, C., 2010. Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. Regul. Toxicol. Pharm. 56, 212–224.
- Maciorkowska, E., Panasiuk, A., Kaczmarek, M., 2005. Concentrations of gastric mucosal cytokines in children with food allergy and *Helicobacter pylori* infection. World J. Gastroenterol. 11 (43), 6751–6746.
- McGaughey, W.H., 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science 229, 193–195.
- National Institute of Allergy and Infectious Diseases, 2006. National Institutes of Health. Report of the NIH Expert Panel on Food Allergy Research.
- Penninks, A.H., Knippels, L.M., 2001. Determination of protein allergenicity: studies in rats. Toxicol. Lett. 2001 (120), 171–180.
- Pilegaard, K., Madsen, C., 2004. An oral Brown Norway rat model for food allergy: comparison of age, sex, dosing volume, and allergen preparation. Toxicology 196, 247–257.
- Sampson, H.A., 1999. Food allergy. Part 1: immunopathogenesis and clinical disorders. J. Allergy Clin. Immunol. 103, 717–728.
- Sayyed, A.H., Haward, R., Herrero, S., Ferré, J., Wright, D.J., 2000. Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a field population of the diamondback moth, *Plutella xylostella*. Appl. Environ. 66, 1509–1516.
- Selgrade, M.K., Kimber, I., Goldman, L., Germolec, D.R., 2003. Assessment of allergenic potential of genetically modified foods: an agenda for future research. Environ. Health Persp. 111, 1140–1141.
- Sicherer, S.H., Munoz-Furlong, A., Sampson, H.A., 2003. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: A 5-year follow-up study. J. Allergy Clin. Immunol. 112, 1203–1207.
- Taylor, S.L., Hefle, S.L., 2001. Will genetically modified foods be allergenic? J. Allergy Clin. Immunol. 107, 765–771.
- Xu, W.T., Cao, S.S., He, X.Y., Luo, Y.B., Guo, X., Yuan, Y.F., Huang, K.L., 2009. Safety Assessment of Cry1Ab/Ac fusion protein. Food Chem. Toxicol. 47, 1459–1465.