Food Research International 64 (2014) 810–821

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Elucidation of immediate type I reactions in native and GM mustard (*Brassica* spp.)

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ARTICLE INFO

Article history:
Received 6 May 2014
Accepted 14 August 2014
Available online 23 August 2014

Keywords:
Allergy
Cytokines
IgE
Mustard
Transcription factors

ABSTRACT

Mustard, a widely consumed spice can provoke allergic manifestations in mustard sensitive individuals. The aim of this study is to explore the allergenicity potential of GM mustard varieties (GM-V2 and GM-V4) having increased carotenoid content and compare it with the native (Varuna) and commercially available variety (Urvashi). Mustard protein sensitized (GM and non-GM) BALB/c mice sera were used to identify the allergenic proteins by IgE immunoblotting. Immunoglobulin levels, mouse mast cell protease-1, monocyte chemotactic protein and histamine were measured in serum. The levels of Th1/Th2 transcription factors GATA-3, T-bet, SOCS3, STAT 6 and c-maf in intestinal proteins of all groups were detected by immunoblotting and PCR. Major IgE-binding proteins of 21, 29 and 33 kDa were found in all mustard varieties. The enhanced levels of Th2 cytokines IL-4, IL-5 and IL-13 and transcription factors GATA-3 and SOCS-3 were observed. The increased levels of MCP-1, MCPT-1 and histamine were also evident in commercial, native, GM-V2 and GM-V4 varieties of mustard treated groups. Conclusively, all these finding indicate that introduction of GM mustard varieties with increased carotenoid content did not cause any increase in allergenicity as compared to its native counterpart and therefore can be safe from allergenicity point of view.

1. Introduction

Globally, the incidence of allergic diseases is on the rise and food allergies constitute a major part of this increase (Harwanegg & Hiller, 2004). The overall prevalence of food allergy is 3–4% in adults and 8% in children (Sicherer & Sampson, 2010). According to US National Institute of Allergy and Infectious Diseases (NIAID 2010) sponsored guidelines, food allergy is an "adverse health effects that arise from specific immune responses on the exposure of a given food" (Boyce et al., 2010). The incidences of food allergy vary among different countries and depend on the consumption habit of a particular country as higher consumption of a particular food may lead to increased sensitization among the susceptible consumers (Dalal et al., 2002).

Oriental mustard *Brassica juncea* (L.) is a member of the Cruciferae (Brassicaceae) plant family. Mustard seeds are used in a variety of food products to enhance flavor and nutritional values. Mustard seeds are composed of protein (23–30%), fixed oil (29–36%), and carbohydrate (12–18%) together with minor constituents including minerals (4%), essential oil (glucosinolates, 0.8–2.3%), phytin (2–3%) as well as phenolic compounds and dithiolethiones. Common foods containing mustard seeds include pickled products, processed meats, seasoning mixes, salad dressings, sauces, and condiments. Foods formulated with mustard seeds are expected to increase in popularity in the future due to its sensory attributes, its high protein content, and its functional properties. However, the widespread use of mustard in foods has raised concerns as mustard can cause IgE-mediated allergic reactions in sensitive individuals (Jorro, Morales, Bras'o, & Pel'aez, 1995). The prevalence of mustard allergy is reported in 1.1% children suffering from food allergies (Morisset et al., 2003; Rance, Dutau, & Abbal, 2000).

Advancements in biotechnology and recombinant DNA technology have resulted in an increasing number of genetically engineered/modified crops. As per different guidelines like Codex Alimentarius, Organisation for Economic Co-operation and Development (OECD), Department of Biotechnology (DBT), India, allergenicity assessment of genetically modified (GM) crops is mandatory prior to their release in the market. Recently, genetic modifications have been introduced in mustard (GM-mustard) by inserting phytoene synthase (*psy*) from *Zea mays* and phytoene desaturase (*crtI*) from *Erwinia uredovora* using Agrobacterium-mediated transformation to increase the carotenoid

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http://dx.doi.org/10.1016/j.foodres.2014.08.014
0963-9969/

content (Al-Babili, Hoa, & Schaub, 2006; Cong et al., 2009). Phytoene synthase (*psy*) and phytoene desaturase (*crtI*) are involved in carotenoid biosynthesis and carotenoids are precursor for vitamin A. These GM mustard varieties with increased carotenoid content were developed to overcome vitamin A deficiency which is a major health problem and may result in blindness and sometimes immune system impairment (Chong & Scheufele, 2002). However, comprehensive safety evaluation is required to assess the allergenic potential of GM mustard varieties.

The aim of this study was to explore the allergenicity potential of GM mustard varieties (GM-V2 and GM-V4) and compare it with the native variety (Varuna) as well as commercially available variety (Urvashi). In order to decipher the role of genetic modification if any, on the allergenicity of GM mustard, many additional allergenicity tests not required by regulatory guidelines have also been undertaken.

2. Materials and methods

2.1. Mustard seeds

Mustard seeds were purchased from a local, certified seed vendor. The name of mustard variety was Urvashi which was produced at Chandra Shekhar Azad University of Agriculture & Technology (CSAUA&T) Kanpur, UP, India and this variety was notified in 2001. GM varieties were obtained as a generous gift from Dr. Vibha Dhawan, The Energy and Resources Institute (TERI), New Delhi, India.

2.2. Mustard crude protein extraction (mustard CPE)

The mustard crude protein extract was prepared as per the previously described method (Misra, Prasad, Das, & Dwivedi, 2009) (Supplementary data S1).

2.3. Animal treatment protocol

Healthy 6–8 week old female BALB/c mice (22 ± 3 g) were obtained from the CSIR-IITR, Lucknow, India animal breeding colony. Animal study protocol (Ref: ITRC/IAEC/09/2012) was approved by the Institute's Ethics Committee of CSIR-IITR, Lucknow. Mice were treated according to the protocol described earlier (Misra et al., 2011) (Supplementary data S2).

2.4. IgE immunoblot analysis

To detect IgE binding proteins in mustard CPE, IgE immunoblotting was performed according to the method described earlier (Misra et al., 2011) (Supplementary data S3).

2.5. Assessment of systemic anaphylaxis score and body temperature measurement

Anaphylactic reactions were scored using the previously described scoring system (Li et al., 2000) (Supplementary data S4). Rectal temperature was measured before and 20 min after the challenge using digital rectal thermometer (Bioseb, France).

2.6. Mustard protein-specific IgE, IgG1 and IgG2a immunoglobulin estimation

Mustard (commercial, native and GM varieties) specific IgE, IgG1, and IgG2a levels were estimated using ELISA according to the earlier described method (Misra et al., 2010) (Supplementary data S5).

2.7. Estimation of total IgE level in mice sera

The levels of total IgE in the mice sera samples of mustard (commercial, native and GM varieties) were determined by the OptEIA™ mouse IgE Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The result was expressed in terms of absorbance.

2.8. Measurement of MCP-1

Levels of monocyte chemotactic protein-1 (MCP-1) in the sera of mustard (commercial, native and GM varieties) treated mice were estimated by ELISA method using a BD OptEIA™ kit (BD Biosciences, San Diego, CA, USA) according to manufacturer's instructions. The result was expressed in terms of absorbance.

2.9. Measurement of MCPT-1 and TSLP

Serum mouse mast cell protease 1 (MCPT-1) and thymic stromal lymphopoietin (TSLP) levels in the sera samples of mustard (commercial, native and GM varieties) treated mice were quantified using ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The result was expressed in terms of absorbance.

2.10. Measurement of PGD_2, cysteinyl leukotriene and histamine

Prostaglandin D_2 (PGD_2), cysteinyl leukotrienes (CysL) and histamine levels in sera of mustard (commercial, native and GM varieties) treated mice were determined using EIA kit (for histamine: SPI-BIO, Montigny le Bretonneux, France; for PGD_2 and CysL: Cayman chemicals, USA) following manufacturers' protocol.

2.11. Histopathological studies

The lungs, intestine and spleen were taken for histological analysis from commercial, native and GM mustard protein treated groups. Tissue sections were stained with hematoxylin and eosin for microscopic examination (125× and 500× magnifications).

2.12. Immunohistochemistry for eosinophil levels

Eosinophil count in the intestine, lungs and spleen sections was performed by immunohistochemistry according to the earlier described method (Li, Sun, Satoh, Fisher, & Spry, 1995) (Supplementary data S6).

2.13. Mast cell staining

To evaluate the number of mast cells in the intestine, lungs and spleen sections, toluidine staining of mast cell was carried out according to the method described earlier (Kumar, Kumar et al., 2013) (Supplementary data S7).

2.14. Splenocyte culture

Splenocyte culture was done according to the method described previously (Yadav, Kumar, Tripathi, & Das, 2013) (Supplementary data S8).

2.15. Immunophenotyping

The level of T-cells (CD4 + and CD8 +) and B-cells in the splenocytes was estimated using flow cytometry according to the method described previously (Yadav, Kumar, Tripathi, & Das, 2013) (Supplementary data S9).

2.16. Semi-quantitative polymerase chain reaction (PCR)

A semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of Th2 cytokines and Th2/Th1 transcription factors (GATA-3, and T-bet) in the intestine of all treated groups was carried out by gene specific primers according to the earlier described method (Kumar, Sharma, Neelabh, et al., 2014) (Supplementary data S10).