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Evaluation of Bar, Barnase, and Barstar recombinant proteins expressed in genetically engineered *Brassica juncea* (Indian mustard) for potential risks of food allergy using bioinformatics and literature searches

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ABSTRACT

The potential allergenicity of Bar, Barnase, and Barstar recombinant proteins expressed in genetically engineered mustard for pollination control in plant breeding was evaluated for regulatory review. To evaluate the potential allergenicity of the Bar, Barnase and Barstar proteins amino acid sequence comparisons were made to those of known and putative allergens, and search for published evidence to the sources of the genes using the AllergenOnline.org database. Initial comparisons in 2012 were performed with version 12 by methods recommended by the Codex Alimentarius Commission and the Indian Council of Medical Research, Government of India. Searches were repeated with version 15 in 2015. A literature search was performed using PubMed to identify reports of allergy associated with the sources of the three transgenes. Potential open reading frames at the DNA insertion site were evaluated for matches to allergens. No significant sequence identity matches were identified with Bar, Barnase or Barstar proteins or potential fusion peptides at the genomic-insert junctions compared to known allergens. No references were identified that associated the sources of the genes with allergy. Based on these results we conclude that the Bar, Barnase and Barstar proteins are unlikely to present any significant risk of food allergy to consumers.

1. Introduction

An evaluation was conducted of the proteins expressed in transgenic Indian mustard lines designed to enable the efficient production of hybrid mustard seeds for improved agronomic traits. This study focused on evaluating potential risks of allergenicity of newly expressed proteins for regulatory review.

1.1. Transgenic Indian mustard lines

Recently, the government of India (GOI) has approved the open field trial release of genetically engineered (GE) mustard, *Brassica juncea* developed by Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi, South Campus (UDSC), New Delhi (Jagannath et al., 2001, 2002; Bisht et al., 2007) with the introduced *barnase-barstar* gene system for pollination control

Abbreviations: AA, amino acid; AOL, AllergenOnline; bp, base pairs; DBT, Department of Biotechnology; GE, Genetically engineered; GM, Genetically modified; GOI, Government of India; ICMR, Indian Council of Medical Research; LB, Left border; ORF, Open Reading Frame; PPT, Phosphinothricin; RB, Right border.

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and heterosis breeding. Two GE mustard events were produced for hybrid production of F1 mustard plants for seed production. The *bar* gene was used as a selectable marker for the selection of both GE events. The genes encoding Barnase and Barstar proteins were introduced into separate transgenic lines to provide male sterility and restorer functions. Protein amino acid (AA) sequences of the three newly expressed proteins are shown in Fig. 1. The transgenic *B. juncea* lines have been developed using *Agrobacterium tumefaceians* transformation (Valentine, 2003). A diagram of the DNA construct inserted in this Barnase mustard line is shown in Fig. 1i) of Jagannath et al. (2001). The construct contains a large DNA spacer with nearly complete genes of two plant proteins that are not expressed due to intentional deletions. The spacer was found to be necessary to restrict expression of Barnase to the tapetum of anthers to prevent adverse agronomic traits (Jagannath et al., 2001). The DNA construct used in the Barstar line is described in Jagannath et al. (2002). The genomic DNA sequences immediately adjacent to the left and right borders of both events were supplied by the developers of the GE mustard. All three novel proteins have been assessed for potential allergenicity by bioinformatics analysis as described here and by assessing the stability of the proteins in the pepsin digestion assay at pH 1.2 in vitro using the method described by Ofori-Anti et al. (2007). Results of the stability in pepsin and stability to heat at various temperatures in vitro have been submitted to the GOI as regulatory studies. In the present paper, the results of bioinformatics analysis of Bar, Barnase, and Barstar proteins are reported along with bioinformatics of hypothetical peptide sequences (Stop-to-Stop coding) defined by computer translation prediction of DNA sequences at the insert border junctions of both transgenic events.

1.2. Food allergy risks

Food allergy is an adverse immune mediated reaction to foods that affects a relatively small percentage of individuals due to complex interactive factors including environmental variation and genetic predisposition that are not completely understood (Lehrer et al., 2002; EFSA, 2010). The majority of food allergies are caused by eight major foods or food groups including peanuts, some tree nuts, cow's milk, hen's eggs, fish, crustacean shellfish, wheat, and soybeans (Metcalfe et al., 1996). Among the various immune *mecha*nisms that are known to cause allergy, IgE mediated immune responses are responsible for most food allergy including severe and life threatening reactions to foods (Burks, 2002). All allergens are proteins but only a small number of proteins, for example the seed storage proteins 2S albumins (Ara h 2, Ara h 6), glycinin (Ara h 3) and beta-conglycinin (Ara h 1) and possibly four low-abundance proteins out of the hundreds of proteins present in peanuts are responsible for peanut food allergy (Porterfield et al., 2009; Peeters et al., 2007). The prevalence of food allergy is relatively low in the general population, ranging from 1 to 5% in adults to 6–8% in children (Thomas et al., 2005; Sicherer and Sampson, 2014). The estimated lifetime prevalence of severe, life-threatening anaphylaxis to foods has been estimated to be between 0.5 and 2.0% in western countries although possibly below 0.1% in some European countries (Beyer et al., 2012). Those are the individuals that should be the primary focus of safety assessments and the primary concern is the potential transfer of a major allergenic protein.

1.3. Assessing the potential allergenicity of GE crops

Assessment of allergenicity of novel proteins expressed in (GE)

a)

>gi|56406595|gb|AAV87646| barnase [Flexi Vector pF1A T7]

1 maqvintfdg vadylqtyhk lpdnyitkse aqalgwvask gnladvapgk siggdifsnr

61 egklqgksgr twreadinyt sgfrnsdril yssdwliykt tdhyqtftki r //

b)

>gi|4775045|emb|CAB42577.1| BARSTAR, [*Bacillus amyloliquefaciens*]

1 mkkavingeq irsisdlhqt lkkelalpey ygenldalwd cltgwveypl vlewrqfeqs

61 kqltengaes vlqvfreaka egcditiils //

c)

>gi|375153556|gb|AFA36668.1| phosphinothricin acetyl transferase [*Streptomyces hygroscopicus*]

1 msperrpadi rrateadmpa vctivnhyie tstvnfrtep qepqewtddl vrlrerypwl

61 vaevdgevag iayagpwkar naydwtaest vyvsprhqrt glgstlythl lksleaqgfk

121 svvaviglpn dpsvrmheal gyaprgmlra agfkhgnwhd vgfwqldfsl pvpprpvlpv

181 tei //

Fig. 1. Amino acid sequences of the three proteins encoded by genes inserted in GM mustard lines to provide male sterility and restorer functions. The inserted DNA sequences and expressed protein sequences were verified by the developers (Jagannath et al., 2001; Jagannath et al., 2002; Bisht et al., 2007). 1a) Barnase, GI|56406595|,111 amino acids as modified from *Bacillus amyloliquefaciens*; 1b) Barstar restorer protein, GI|4775045|, 90 amino acids from *Bacillus amyloliquefaciens*; 1c) Bar (phosphinothricin acetyl transferase), GI|375153556|, from *Streptomyces hygroscopicus*, 183 amino acids.

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