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Bioinformatic analysis for allergenicity assessment of *Bacillus thuringiensis* Cry proteins expressed in insect-resistant food crops

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ABSTRACT

The novel proteins introduced into the genetically modified (GM) crops need to be evaluated for the potential allergenicity before their introduction into the food chain to address the safety concerns of consumers. At present, there is no single definitive test that can be relied upon to predict allergic response in humans to a new protein; hence a composite approach to allergic response prediction is described in this study. The present study reports on the evaluation of the Cry proteins, encoded by *cry1Ac, cry1Ab, cry2Ab, cry1Ca, cry1Fa/cry1Ca* hybrid, being expressed in *Bt* food crops that are under field trials in India, for potential allergenic cross-reactivity using bioinformatics search tools. The sequence identity of amino acids was analyzed using FASTA3 of AllergenOnline version 10.0 and BLASTX of NCBI Entrez to identify any potential sequence matches to allergen proteins. As a step further in the detection of allergens, an independent database of domains in the allergens available in the AllergenOnline database was also developed. The results indicated no significant alignment and similarity of Cry proteins at domain level with any of the known allergens revealing that there is no potential risk of allergenic cross-reactivity.

1. Introduction

With the advent of genetic transformation technology, it has become possible to incorporate *cry* genes and thus, to express *Bacillus thuringiensis* (*Bt*) Cry proteins in plant cells so that target insect larvae infesting the crop plants are effectively killed. The Cry proteins encoding delta-endotoxins from *B. thuringiensis* provide protection against a wide range of lepidopteron and dipteran insect pests throughout the growing season of the plant. The first *Bt* crops, *viz*., *Bt* cotton, *Bt* corn and *Bt* potato were commercialized in USA in 1996. Currently, *Bt* crops are cultivated in 23 countries over an area of 46 mha. In India, till date, six events of *Bt* cotton, i.e., MON531 with *cry1Ac* gene, MON15985 with *cry1Ac* and *cry2Ab* genes, GFM Cry1A with fused *cry1Ac* and *cry1Ab* genes, Event 1 with truncated *cry1Ac,* Dharwad event with truncated *cry1Ac* gene and event 9124 with *cry1C* gene have been commercialized, which are being cultivated in an area of 8.4 million hectares (James, 2009: Choudhary and Gaur, 2010).

Prior to the introduction of the GM crops into the market, their safety needs to be thoroughly scrutinized for their potential allergenicity. The various components of the allergenicity assessment recommended by the Codex Alimentarius Guidelines include the source of the protein, sequence similarity with known allergenic proteins, *in vitro* digestibility and degradability, sera binding tests, animal models and clinical tests. However, ethical concerns may be raised over these clinical or immunological tests as they may expose patients to harmful substances that may elicit adverse reactions. A 'weight of evidence' approach including bioinformatics, digestibility and animal models is recommended by the Codex Alimentarius Guidelines to assess the risk of allergenicity of GM foods (<http://library.wur.nl/frontis/allergy_matters/10_kleter.pdf>).

Among the various strategies employed for assessing the potential allergenicity, the prerequisite is *in silico* sequence analysis (Stadler and Stadler, 2003). To advance the strategy for allergy-related safety assessment of genetically transformed foods a revised decision tree approach was set forth by the Food and Agricultural Organization/World Health Organization (FAO/WHO) of the United Nations. The decision tree (FAO/WHO, 2001b) with suggested modifications to include the determination of domain similarity is presented in Fig. 1. This decision tree has increased the rigor in the evaluation of amino acid sequence homology from eight amino acids down to six amino acids, along with a 35% identity match over any 80 or more contiguous amino acids throughout the sequence of the protein (Metcalfe, 2005). Sequence homology between the proteins is likely to cause cross-reactions. However, structures with low sequence similarity may also cause cross-reactivity due to the fact that the structural similarity is possible even in absence of sequence similarity (Opiyo and Moriyama, 2007).

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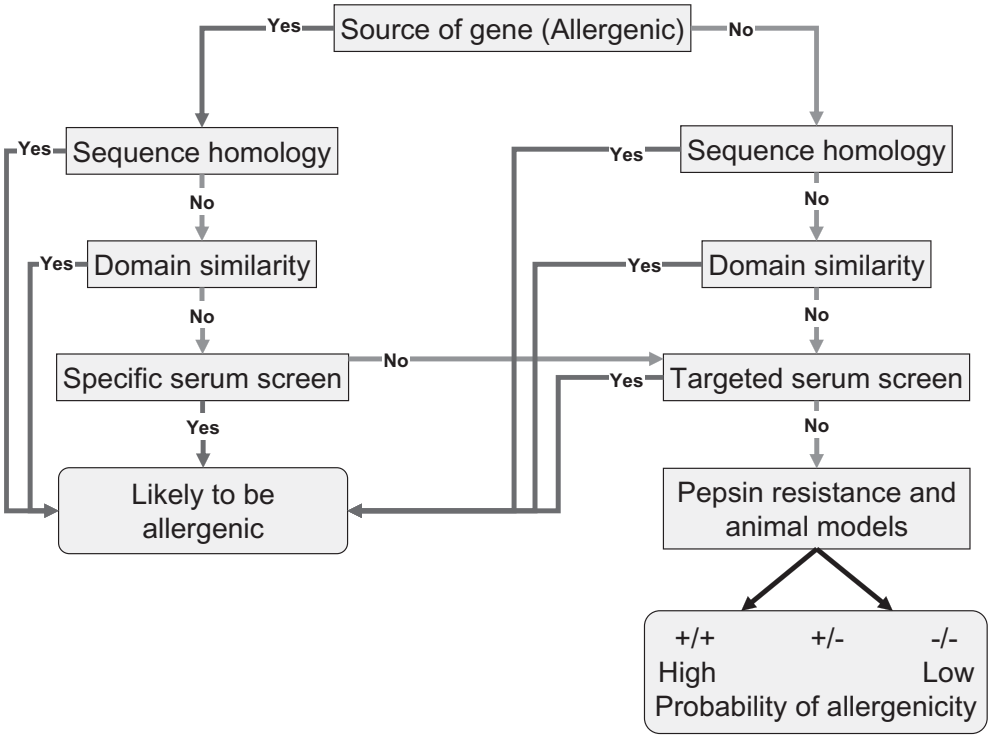

Fig. 1. Modified version of proposed step-by-step 'decision tree' assessment for allergenicity of GM crops. 2001 revised decision tree set forth by a FAO/WHO consultancy. *Source*: Metacalfe (2005).

Allergens are proteins that elicit powerful T helper lymphocyte type 2 (Th2) responses, culminating in Immunoglobulin-E (IgE) antibody production and the development of allergic conditions. Allergic reactions consist of a series of events that start with recognition of the native allergen structure by antigen presenting cells and culminate in IgE antibody production and mast cell sensitization and triggering (Furmonaviciene et al., 2005). According to the state of the art, there are two classes of food allergens (Fig. 2): class 1 allergens are capable to sensitize as well as to trigger allergic reactions via the oral route. Whereas class 2 food allergens do not sensitize orally because they are easily digested and thereby lose their sensitization potential. Thus, they trigger IgE mediated reactions only at the primary contact sites. *In vitro* digestion tests used as decision criteria by International Food Safety Authorities are suitable tools for revealing class 1 food allergens, but they do not permit a safety classification of digestion-labile class 2 proteins in settings of stomach insufficiency (Jensen-Jarolim and Untersmayr, 2006). Allergens are resistant to proteolytic digestion, i.e., resistant to those enzymes that are present in gastrointestinal (GI) tract that normally act to digest the large proteins to smaller ones. Pepsin is the main proteolytic enzyme in the GI tract and hence, pepsin has been utilized to test the stability of allergenic proteins.

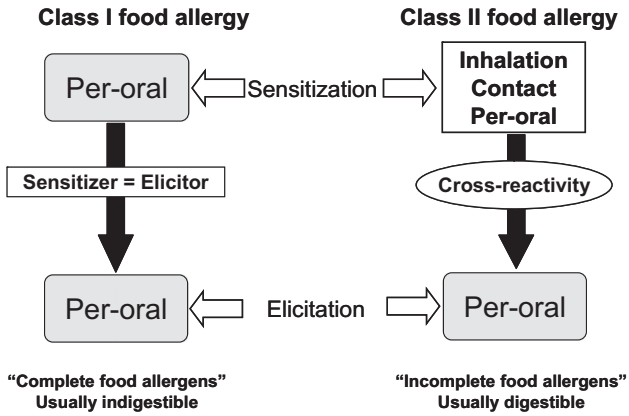

Fig. 2. Comparison of class I food allergy (left) and class II food allergy (right). Class I food allergy is caused by complete food allergens that function as a per-oral sensitizer as well as a symptom elicitor. Complete food allergens are usually resistant to heat and digestive enzymes. Class II food allergy is caused by incomplete food allergens, which cannot easily establish per-oral sensitization because of their sensitivity to heat or digestion. These incomplete allergens provoke allergic reactions in already-sensitized patients based on their cross-reactivity to the corresponding sensitizers. *Source*: Yagami (2002).

In addition to above facts, it has been proposed that proteolytic activity is adjuvant for allergenicity, however, lipid binding is more common and is found for more than 50% of the major allergens. Such structures can enhance allergenicity via Toll-like receptors (TLRs) and CD1 pathways (Thomas et al., 2005). Toll-like receptors (TLRs) comprise an important and interesting group of receptors that regulate pathogen-related responses and the subsequent immune response. TLR signaling can enhance both Th1 and Th2 responses and be induced by peptides as well as non-proteinaceous ligands. Another common feature of protein allergens is that they tend to be glycosylated. Some allergenic proteases may partially act through activation of the protease-activated receptor 2 (PAR2). PARs are family of proteolytically activated G-protein couple receptors (Wills-Karp et al., 2010). Therefore, allergic reactions can be induced through proteolytic activity, engagement of pattern recognition receptors, molecular mimicry of TLR signaling complex molecules, lipid-binding activity and oxidant potential.

The primary consideration in allergenicity assessment of a newly expressed novel protein in a food product derived from GM crop is the prevention of unexpected exposure of sensitized individuals to food allergens. This includes the assessment of the potential for foods containing such novel proteins to cross-react with known food allergens or to lead to the development of *de novo* hypersensitivity