An Approach to Reconfirm Transgenic "Cry" Protein Sequences as Safe for Use in Genetic Engineering by Bioinformatic Tools

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Abstract: ***Background*:** Genetically engineered (GM) crops are produced by the insertion of specific genes from *Bacillus thuringiensis* [Bt] that encode a transgenic protein which must be evaluated for potential safety and allergenicity prior to crop development and market release.

***Objective*:** The aim of the present work was to re inspect the allergenicity of Cry 1Ab, Cry 1Ac and Cry 1C transgenic protein sequences using FASTA based bioinformatics tools.

***Methods*:** An *in silico* approach was employed to assess allergenicity and cross-reactivity of three Cry proteins- Cry 1Ab, Cry 1Ac and Cry 1C being preferred transgenic proteins of crop developers in India. A non-allergenic dietary spinach rubisco, a small subunit protein, and a known food allergen Arah 1 were analysed as per recommended criteria, using Full FASTA alignment and 80 amino acid window approach in allergen databases- FARRP and SDAP.

***Results*:** None of the transgenic Cry 1Ab, Cry 1Ac and Cry 1C proteins showed sequence similarity of >35% with any known allergenic sequence in allergen databases. Dietary protein showed a high of only 21% similarity with Apim allergen sequence, while Arah 1, a proven food allergen reflected greater than 35% sequence similarity with known allergen such as beta-conglycin under the 80 amino acid window approach.

***Conclusion*:** The allergenicity assessment by *in silico* tools of three Cry proteins, used for development of genetically engineered crops did not indicate significant alignment and similarity with any known allergen(s) in the database. This confirmed the approach for use of Cry proteins as safe transgenic proteins in genetic engineering from allergenicity point of view.

Keywords: Cry proteins, *Bacillus thuringiensis* (Bt), Allergenicity, Bioinformatics, FASTA.

INTRODUCTION

Conventional farming practices, low yield, crop loss each year due to insect pests and weeds, drought conditions, and others are always of great concern in the development of agriculture and progress of a nation's economy. To address these major concerns, Agricultural Biotechnology more specifically plant genetic engineering, is a major revolution in agriculture worldwide [1]. Transgenic engineering involves the deliberate transfer of genes between species (or within species) by using bio-techniques, which lead to production of GM crops. One of the first—and still the most widespread— uses of GM crop technology is the development of crops resistant to common insect pests through genetic modification with genes obtained from the soil bacterium *Bacillus thuringiensis* which has been reported to produce crystal / Cry proteins or inclusion bodies with insecticidal activity [2].

The primary focus for the assessment of the potential allergenicity of a GM crop is the bioinformatic comparisons of proteins introduced into food crops through genetic engineering versus those of known allergens. This provides an efficient mechanism to identify the proteins that may present an increased risk of allergic reactions for individuals with existing allergies [3]. The transfer of genes from common allergenic foods is discouraged, unless it can be documented that the gene transferred does not code for an allergen. This is evident from Brazil nut allergen (2S albumin) controversy, which was intended for over expression in soybean, but was found to retain its allergenicity and was therefore withdrawn from commercialization [4].

Under the framework designed for allergenicity and safety assessment of GM crops by various international and national regulatory organizations, amino acid sequence homology has been stressed upon before GM seed development and its commercial release. Bioinformatics based amino acid sequence homology comparisons assess the extent to which a newly expressed protein (regardless of the source) in GM crops is similar in structure or share cross-reactivity to known allergens [5,6]. Aalberse [7] argued for a match of more than 50 – 70 % identity over the full length sequence alignment is much likely to indicate potential

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in vitro cross – reactivity [7] Sequence identities greater than 35% in a segment of 80 or more amino acids are always true positive searches, for suspecting the potential allergenic cross-reactivity of recombinant proteins [8].

The aim of the present work was therefore, to reinvestigate three Cry protein sequences – Cry 1Ab, Cry 1Ac and Cry 1C, used in the development of GM crops by FASTA based *in silico* approaches for allergenicity in allergen specific databases.

MATERIALS AND METHODS

A number of transgenic protein sequences such as – Cry 1A, Cry 1Ab, Cry 2Ab, Cry 1Ac, Cry 1C have been incorporated in the development of Bt cotton, the only commercialsed GM crop in India. A few of these sequences have been selected for developing GM food crops such as maize, rice, cauliflower, cabbage for imparting pesticide resistance as per reports of IGMORIS [9]. We therefore selected Cry 1Ab, Cry 1Ac and Cry 1C transgenic protein sequences for investigating allergenicity through two FASTA based approaches as – full length search and short 80 amino acid window search. Simultaneously, as control, a proven peanut allergen sequence Ara h1 and a commonly consumed non allergenic spinach small subunit protein as negative control, sequence was also assessed for allergenicity properties [2].

Selection of the Query Protein Sequences

The three protein sequences Cry -1Ab, 1Ac, 1C (Accession No - P0A370, P05068, Q58FM0), Peanut allergen Arah 1(P43237) as positive sequence and spinach rubisco small subunit protein, SSU(P00870) were analysed in the present work. The sequences were retrieved from UniProt (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov) databases.

Allergen Database Selection

Sequence homology search was performed against two allergen specific databases – FARRP, Allergen Online (http://www.allergenonline.com) and Structural Database of Allergenic Proteins (SDAP), (http://fermi. utmb.edu) [10].

Full Length FASTA Search

FASTA comparisons are performed by aligning the query sequence as first match of a specific word size (ktup=2), followed by extension of alignment. Specific parameters considered in this tool includeed an expectation threshold=10, a gap creation penalty =12 and gap extension penalty = two. FASTA algorithm employs BLOSUM matrix series, which is derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. The BLOSUM50 matrix identified blocks of conserved residues, that were at least 50% identical. The extent of similarity was calculated as percent similarity. The query sequence, if observed for sharing identity > 50 % under Full length FASTA alignment, with known allergens was considered as allergenic sequence.

80 Amino acid Sliding Window Approach

FASTA alignment was carried out to compare the possible contiguous amino acid segments of each of the five query proteins against the listed sequences in the databases. Each amino acid sequence of individual protein was searched starting with 1–80 aa, then 2–81 aa, and so on until the last 80 amino acid segment of each protein is compared with database. In this search, percent identities were calculated to evaluate possible cross allergenicity. An alignment of query sequence showing >35% similarity over segments as short as 80 amino acids with known allergen(s) indicated that the query sequence might be a potential allergen and should be subjected to further *in vivo* and *in vitro* testings [2].

RESULTS

Sequence Search Analysis of Cry Proteins Under SDAP Database

The maximum similarity in full-length alignment, for the three cry proteins of interest, was obtained as percentage similarity in the range of 6-4% only, whereas in case of 80 amino acid window approach highest sequence identity of less than 35% was observed. We described here the top matched sequences (3 – 4) under Full FASTA alignment and 80, amino acid window approach (Table **1**).

The Full FASTA alignment of the Cry 1Ab protein sequence yielded highest sequence similarity of only 6% with Aspf5, a fungal allergen from *Aspergillus fumigatus*, 2 % with Mala s 1, a fungal origin allergen from *Malassezia synpodialis*, Tria gliadin from *Triticum aestivum* and Pench 20 from *Penicillium chrysogenum*. The 80 amino acid sliding window showed 32% identity (26 over 80 amino acids) with Mala s 1 and 27% identity with food allergen *Gly m* 1 of *Glycine max* and 26% with pollen allergen, Ligv1 of *Ligustrum vulgare*.

Table 1: Sequence Homology Analysis for Cry Proteins and Control Sequences Using FASTA Against Allergen Databases

Query Sequence	Length (aa)	Accession No.	Full length FASTA Alignment % Similarity/ E score			80 amino acid Sliding window Approach, % Similarity		
			SDAP		Allergen Online	SDAP		Allergen Online
Cry 1Ab	1155	P0A370	6%	Aspf 5 (CAA83015)	No sequence with E score < 1.00	32%	Mala s1 (Q01940)	No hits > 35% identity found
			4%	Mala s1 (Q01940)		27%	Glym1 (AAB09252)	
			2%	Pen ch 20.0 (AAB34785)		26%	Lig v1 (O82015)	
Cry 1Ac	1178	P05068	3.7%	Tri a gliadin (AAA34285)	No sequence with E score < 1.00	30%	Asp f13 (P28296)	No hits > 35% identity found
			3.6%	Ana c 2 (BAA21849)		25%	Lig v1 (O82015)	
			2.7%	Phl p 5.0 (CAD87529)		23%	Tria (AAA34285)	
Cry 1C	1044	Q58FM0	4.7%	Hev b 9 (Q9LEJ0)	E score = 0.9 with 29 kD IgE binding protein from *Candida albicans*	33%	Cora 1(CAA96549)	No hits > 35% identity found
			4%	Canda 3 (AAN11300)		27%	Cand a 3 (AAN11300)	
			3%	Pas n s1 (ACA23876)		26%	Chaf 1(Q9N2R3)	
Ara h 1	614	P43237	Lenc 1 1.1e – 68		Lenc 1 (Q84UI0) 4.5e - 068	Ara h 1		Vicilin 65%
			Lupan 1 4.2e – 56		Lupan 1, Conglutin beta (B8Q5G0) 2.4e - 056			Lupan 1, Conglutin beta 63%
			Gly m Conglycinin 2.9e – 51		Beta Conglycinin, *Glycine max*. (AAB23463) 1.2e - 026			Len c1 63%
Spinach SSU	123	P00870	Can f 3(AAB30434) 14%		NIL	Apim 10 21%	Horv 12 15%	NIL
			Apim (ABF21077) 14%					
			Tria 12 (P49232) 11%					

The highest scoring identity for the Cry 1Ac protein sequence under Full FASTA alignment was found to be nearly 3 percent with different proteins namely Tria gliadin, Anac 2 from *Ananas comosus* and 2.7% with Phlp 5.0 allergen from timothy grass. Protein sequence of Cry 1Ac under 80 amino acid window reflected a highest of 30% identity with Aspf 13, an alkaline proteinase from *Aspergillus fumigatus*, 25 % with Lig v1 and 23% with Tria gliadin similar as also observed with respect to Cry1Ab.

The maximum scoring similarity as observed under Full FASTA alignment for Cry 1C protein sequence was found to be nearly 4% with known allergen(s) as Hev b 9 of *Hevea brasiliensis*, peroxisomal protein Canda 3 from *Candida albicans* and ~ 3% with Pasn 1,grass allergen. In the 80 amino acid window approach, a highest identity of 33.7% with Cor a1, pollen allergen from *Corylus avellana,* 27% with Cand a 3 and 26% with Chaf 1 tropomyosin from crab, *Charybdis feriatus* and Pasn 1, a beta expansin protein of Poales family was observed.

The Bioinformatics Assessment of Cry Proteins Using FASTA v35 Against the FARRP Allergen Online

Neither of the Cry 1Ab, Cry 1Ac and Cry 1C protein sequences shared sequence similarity of the value of E score > 1 with known allergenic sequences in the database. In case of, Cry 1C protein a sequence identity with E score = 0.9 and similarity as 26%, with 29 kD IgE binding protein from *Candida albicans* was reflected which is well below the threshold of > 50 % to be classified as allergenic sequence (Table **1**).

Sliding 80mer window approach of the FARRP for the Cry 1Ab, Cry 1Ac & Cry 1C didn't present any segment with over 35% similarity to any known allergen. The results did not meet the criteria of Codex [5] for positivity of allergenic cross reactivity as well as sharing greater than 35% similarity over 80 amino acids against known allergenic sequences for Cry proteins.

Apart from transgenic proteins under evaluation, known peanut allergen Ara h 1(P43237) was analysed as positive control across two allergen databases. Comparison of the 614 aa long sequence of Arah 1 against AllergenOnline resulted in Full length FASTA 85% sequence identity with Len c 1.0 from *Lens culinaris* (E score=4.5e - 068) and Glym Conglycin (E score = 1.2e – 026)) from *Glycine max* (Table **1**). The 80 amino acid sliding window search showed that Arah 1 shares identity greater than 35% with known allergens as beta- conglycin from *Glycine max.*, Len c 1 from *Lens culinaris*, Vicilin from *Pisum sativum* and Lupan 1 from *Lupinus angustifolius*. Spinach rubisco small sub unit (SSU) protein is a 123 aa long (P00870) sequence, served as negative control. Under the full FASTA alignment, a sequence identity of 14% with Canf3 from *Canis familaris* and Apim from *Apis mellifera* was observed in SDAP, followed by highest of 21% identity with Apim over 80 aa window approach. A similarity with known allergenic sequence was lacking in AllergenOnline database analysis.

DISCUSSION

The application of conventional agricultural chemicals and pesticides can reduce insects and weed manifestation but many consumers are concerned about the safety of pesticide residues in their foods and the potential adverse environmental effects associated with these practices [1]. From a consumer perspective, GM crops hold great promise for foods with improved quality characteristics. Safety and allergenicity of GM crops need to be evaluated before commercial release as recommended by Codex [5] as well as suggested by each country' s defined regulatory guidelines. One of the important prerequisite of the allergenicity assessment that should begin early in the development of GM crop/plant is bioinformatic comparison of transgenic proteins (regardless of the source) to those of known allergens by FASTA or BLAST algorithms to determine for any sequence identity causing allergic reactions as reported and supported by various authors [2,11].

Codex have stressed on short stretch search of 35% identity over 80 amino acid as a conservative prediction for allergenicity assessment instead of using short contiguous six amino acid window approach which results in false positive results [5, 12, 13]. A study on identification of allergenic sequences in 6 *Bacillus thuringiensis* insecticidal proteins, including Cry 1Ab and Cry 1Ac proteins, against a assembled allergen and gliadin sequence database, named ALLERGEN3, have been reported. By using short 6-8 contiguous window amino acids approach, the highest identity demonstrated for Cry 1Ab and Cry 1C proteins was found at 5 and 6 contiguous identical amino acids respectively, which is still below the threshold of minimum 8 and to be identified as constituting the so called true linear or continuous IgE binding epitopes [2]. In another study in 2002 [14] revealed a potential IgE – binding linear epitope of allergens in 33 transgenic proteins, including 7 Cry protein sequences. Using the 6 amino acid window size approach, as well as Hopps and Wood prediction method, this study indicated that Cry 1Ac shared two identical peptides, GNAAPQ and GSTGITI with cedar pollen allergens. However, Hopp and Wood's prediction method did not indicate pronounced allergenicity for the GNAAPQ sequence in the cedar pollen allergens and yielded a negative score for the GSTGITI sequence. This further confirmed Cry 1Ac as non allergenic sequence and also supported the importance of 8 amino acid window size approach as also advocated by Hileman *et al.* [2, 14].

In the past few years, limited studies have been reported based on *in silico* approaches for assessment of transgenic proteins being used for development of GM crops in India. Verma and coworkers [15]. reported for validation of safety of Cry 1Ab and Cry 2Ab transgenic protein sequences using 8-mer, 80-mer, and full FASTA approach against AllegenOnline database and concluded them as safe for use in plant genetic engineering. In another study by Randhawa *et al*. [16] for evaluation of various Cry proteins as Cry1Ac, Cry1Ab, Cry2Ab, Cry1Ca, Cry1fa/Cry1Ca, which are being incorporated in *Bt.* Crops in India (under development), the sequence identity of Cry protein sequence (amino acids) was analyzed using FASTA3 of AllergenOnline version 10.0 and BLASTX of NCBI Entrez and were also compared against an independently developed allergen domain database "Interproscan" to identify any potential allergenic sequence. The stated bioinformatics searches did not indicate any significant alignment nor similarity of Cry proteins at domain level with any of the known

allergens revealing that there is no potential risk of allergenic cross-reactivity

We aimed at analysing the three transgenic proteins – Cry 1Ab, Cry 1Ac and Cry 1C, being among the preferred transgenic proteins for development of GM crops in India, for their potential allergenicity and cross-reactivity by bioinformatics search as per the guidelines of Codex [5]. The Cry 1Ab and Cry 1Ac protein sequences did not fulfill the criteria of Aalbrese, [7] of having full length FASTA score of greater than 50% to be suspected as allergenic sequences sharing cross-reactivity or IgE binding with reported allergens in the specified allergen database as SDAP and AllergenOnline. The same Cry 1Ab and Cry 1Ac protein sequences under 80 amino acid homology approach did not present any sequence with greater than 35% similarity for suspecting any allergenicity in accordance with the earlier studies employing AllergenOnline databases [2, 15, 16].

The Cry 1C transgenic protein sequence is also found to be non allergenic as no significant similarity with any known allergenic sequences in FARRP and SDAP allergen databases was observed. Similar to Cry 1Ab and Cry 1Ac, the Cry 1C transgenic protein also did not indicate any sequence identity > 35%under 80mer sliding window approach to be declared as showing allergenicity or sharing cross-reactivity [16].

The peanut (a legume) protein Ara h1, selected as positive control was also analysed for sequence homology in the specified allergen databases. The protein sequence of Arah 1 indicated sequence similarity with E score < 1 and greater than 85% similarity under full FASTA alignment with reported major food allergens of the same legume family. Under the 80 amino acid window, Ara h 1 is observed to reflect identity of greater than 60% (minimum threshold of > 35% required to be classified as allergen) with well known food allergens such as Conglutin β and vicilin family of seed storage protein [17, 18]. The same is found in legume seeds in the AllergeOnline database analysis while the same Arah1 protein sequence was observed to show sequence homology with its isoforms in the SDAP database. The present sequence homology analysis for Arah 1 is another way of reconfirming it as an allergen on the basis of bioinformatic approaches, as major IgE binding epitopes of Ara h1 have already been deciphered and reported by Shin *et al.* [19] and Horihane *et al.* [20]. Ara h1 is a well documented major allergen of peanut by immunochemical assays [21].

The full length FASTA alignment of the Spinach small sub unit sequence, a dietary protein which served as a negative control in this study did not present any similarity with known allergens nor exhibited any possibility of cross-reactivity as no single matched sequence > 35% similarity in 80 aa window approach in the AllergenOnline and SDAP databases was observed. Hileman *et al.* have also confirmed for non-allergenic nature of the same protein by using window size of 5 – 8 amino acids against the AllergenOnline database [2].

In conclusion, the bioinformatics analyses were performed for the transgenic proteins expressed in GM food crops that are under development or confined field trials in India. The results obtained reconfirms that none of the three described Cry proteins were found be positive for potential allergenic cross-reactivity or sequence similarity and were ascertained to be safe from allergenicity point of view. Thus, the Cry group of proteins appeared as safe transgenic proteins for use in plant genetic engineering.

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ABBREVIATIONS

GM	=	Genetically modified
Bt	=	*Bacillus thuringiensis*
FARRP	=	Food Allergy Research and Resource Program
NCBI	=	National Centre for Biotechnology Information
BLAST	=	Basic Local Alignment Search Tool
IGMORIS	=	Indian GMO Research Information System
GMO	=	Genetically modified Organism

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