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In silico allergenicity evaluation of cry proteins of *Bacillus thuringiensis* expressed in Bt-Brinjal (*Solanum melongena* L.)

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A B S T R A C T

Prior to commercial production, it is necessary to assess the biosafety of the novel recombinant proteins added to Bt-Brinjal (*Bacillus thuringiensis*) with regard to their probable allergenicity. In the Bt-brinjal plant, the cry1Ac, cry1Ab, and cry1Fa1 genes produce toxic proteins that are highly lethal to insects, particularly lepidopteron insects, and offer defence against the fruit and shoot borer (FSB), also known as *Leucinodes orbonalis*. However these proteins are inactivated in acidic environment of the human and animal gut, so they are not toxic. In the current study, cry1Ac, cry1Ab, and cry1Fa1 proteins produced in Bt-Brinjal were assessed for their probable allergenic cross-reactivity by sequence comparison using bioinformatics techniques. *In silico* assessment, these recombinant proteins expressed in Bt-Brinjal were compared for homology using online databases (Allergen Online version 21.0, AlgPred, and SDAP), BLASTX program of NCBI and FASTA. Sequence alignment with allergen proteins was performed using three approaches: complete sequence, 80 amino acids, and 6-8 amino acids. In addition, the cry proteins were examined for the existence of domains that matched those of the allergen proteins to determine whether allergen cross-reactivity was likely even in the absence of sequence homology. Sequence alignment using the three approaches above and at the epitope level shows no similarity of cry1Ac, cry1Ab, and cry1Fa1 to any of the known allergen proteins. Since the potential cross-reactivity of these recombinant proteins with allergen proteins was not reached, this result may help farmers, researchers, and policy makers to promote the cultivation of Bt-brinjal in terms of biosafety.

Introduction

Advances in genetic modification have made it possible to develop genetically modified food crops by transferring novel genes into crops that are resistant to herbicides, pests, and water and salt stress. In insect-resistant food crops, Cry proteins from *Bacillus thuringiensis* (Bt) are transferred to crops to protect them from various insect species. Delta endotoxins encoded by the Cry proteins protect against a wide range of Lepidoptera and Diptera pests. This transformation of Bt-Cry genes has been made in many food crops, namely Bt-Brinjal, Bt-Rice, and Bt-Potatoes. A cry gene (cry1Ac) is inserted into the genome of a brinjal cultivar to create Bt-Brinjal (Bt eggplant), which offers resistance to lepidopteran insects, particularly *Leucinodes orbonalis*. Agrobacterium-mediated gene transformation technique is used to insert the cry1Ac gene into the brinjal plant alongside other genetic elements such as an antibiotic resistance marker gene, promoters and terminators. Further transformations of other Cry genes such as cry1Ab, cry1Fa1 into brinjal plants are currently being tested to investigate efficacy against Lepidoptera insects. Before being introduced into the food chain and the market, the biosafety of the genetically engineered food crops must be assessed for toxicity and allergenicity. Immunoglobulin E (IgE)-recognized allergens are proteins that interfere with a person's immune system. Most frequently, allergenic proteins attach to specific IgE molecules on the surfaces of mast cells and basophils to trigger an immune response (Kindt *et al.,* 2006). Allergenicity assessment is one of the most crucial factors when evaluating the biosafety of genetically modified plants (GMPs). Digestibility, bioinformatics analysis, and animal models are some of various methods of allergenicity assessment suggested by the Codex Alimentarius recommendations for allergenicity assessment of GM plants (Randhawa *et al.*, 2011). Bioinformatics analysis is considered an important method for analyzing the safety evaluation of genetically modified plants using a database search. Database searches to ascertain the allergenicity of novel proteins are encouraged by the World Health Organization (WHO), the European Food Safety Authority (EFSA), and the United States Environmental Protection Agency (Ivanciuc *et al.,* 2011; Kok *et al.,* 2008). More than 2000 allergens have been found and are listed in various allergen databases as of now. Using similarity search techniques, these databases have been extensively used to find sequence similarities with the probable allergens that are available in the database (Allahyari *et al.,* 2013). For determining whether recombinant proteins synthesized from transgenes of genetically modified plants are allergenic, several allergy databases and computational methods are becoming more and more crucial (Fard *et al.,* 2013). A number of databases, including FARRP, SDAP, ADFS, PSD, Allergome, and Algpred, have been proposed for *in silico* identification of allergens. The application of bioinformatics tools and techniques for the assessment of potential allergenicity of genetically engineered novel proteins has significantly risen in the last few decades (Ladics, 2008). Using sequence alignment searches and bioinformatics tools and techniques, the cry1Ac, cry1Ab, and cry1Fa1 proteins expressed in Bt-Brinjal were examined for their probable allergenic cross-reactivity. The National Center for Biotechnology Information's (NCBI) BLASTX program, FASTA, and online databases (Allergen Online version 21.0, AlgPred, and SDAP) were used for *in silico* assessment, or similarity searches of these recombinant proteins expressed in Bt-Brinjal. The cry proteins were examined for the existence of domains that matched those of the allergen proteins to determine whether allergen cross-reactivity was likely even in the absence of sequence homology.

Materials and Methods

Query protein sequences taken for the study

For this investigation, the cry1Ac, cry1Ab, and cry1Fa1 genes of *Bacillus thuringiensis* were employed. The translated protein sequences of these cry genes, cry1Ac, cry1Ab, and cry1Fa1 (GenBank accession numbers: Y09787, X5493, and M73254,

respectively), were deposited in FASTA format for future research. The nucleotide sequences of these genes were retrieved from the NCBI GenBank database.

In silico assessment of the genetically modified proteins expressed in Bt-Brinjal using online databases

Numerous databases are available for *in silico* assessment of genetically modified proteins expressed in Bt-Brinjal. Three databases were used for this study, namely Allergen Online version 21.0 (http://www.allergenonline.com), Algpred (http://crdd.osdd.net/raghava/algpred/), SDAP (https://fermi.utmb.edu/SDAP/).

The Allergen Online Version 21.0 database

This database, which can be accessed at <http://www.allergenonline.org> is a useful resource for pinpointing the primary potential allergenic issues for GMOs (genetically modified organisms) and novel foods in accordance with the guidelines set forth by the Codex Alimentarius Commission (2003). The Allergen Online database contains 2233 sequence entries of allergenic proteins derived from a variety of sources, including food, respiratory, contact, and toxic allergens. A protein sequence is compared with entries in the allergy database using FASTA on this website (Pearson and Lipman, 1988). The protein sequence is searched in this database using three distinct approaches, including checking for full-length alignments using FASTA, which shows high identity score alignments and hint to the probability of allergic cross-reactivity. FASTA results confirm that when a protein shares more than 70% of its entire length with an allergenic protein, it has a higher chance of being cross-reactive. If the protein shares less than 50%, it has a chance to be less likely cross-reactive and unlikely to be cross-reactive. Search with 80-mer sliding window, each query protein having each feasible adjacent 80-amino acid sequence were looked up, starting with first amino acid position 1-80, moving on to 2-81, 3-82, and so forth until each protein's final amino acid section was evaluated (Aalberse, 2000). A search for an exact match of 8 amino acids should be conducted, with a search for 8-amino acid short sequence identity intended for an extremely cautious search, and alignments showing > 35% identity across segments of up to 80 amino acids should be screened for a protein to know probable cross-reactivity(Alimentarius,2003).As of now scientific community has found no evidence that a protein that matches an 8-amino acid sequence is more probable to be cross-reactive and might be unnoticed by the conservative 80-amino acid match (35%).

Algpred

AlgPred is available at <http://crdd.osdd.net/raghava/algpred/>. AlgPred is a web server created to anticipate allergenic proteins and allergenic areas in a protein. To reliably predict allergenic proteins, a deliberate effort has been made in AlgPred to integrate a variety of approaches (Saha & Raghava, 2006).

Structural Database of Allergenic Proteins (SDAP)

Access to SDAP is available free of charge from <https://fermi.utmb.edu/SDAP>.This database combines allergenic proteins with different computational tools and techniques to support studies on the structural biology of different allergens. SDAP is a crucial tool for investigating the probable cross-reactivity between identified allergens including cross-checking of novel proteins for conformity with the FAO/WHO allergenicity recommendations and forecasting the IgE-binding capability of genetically engineered food proteins. With help of this browser-based Internet service, it is possible to locate an allergen's sequence as well as structural neighbours, search for an epitope presence, as well as retrieve information about an allergy from SwissProt, PIR, NCBI, PDB databases. The FASTA 3.45 tool is used in SDAP to perform alignments between the query protein sequence and all SDAP allergens. E values > 0.01 signify that there is no homology between the query protein sequence and the database sequences, while

alignments with E values < 0.01 usually invariably show homology.

In silico evaluation of genetically engineered proteins expressed in Bt-Brinjal using NCBI's BLASTX tool

The genetically modified proteins synthesized in Bt-Brinjal were examined for allergenic cross-reactivity using the BLASTX tool, which is run by NCBI (National Center for Biotechnology Information). To find homologous sequences of the allergen protein, the BLASTX tool was used with the cry1Ac, cry1Ab, and cry1Fa1 genes of *Bacillus thuringiensis*.

Comparison of the allergen and cry protein domains

The domains in cry1Ac, cry1Ab, and cry1Fa1 proteins were searched using InterProScan, which is available at The European Bioinformatics Institute web server. The cry protein domains were matched to those in the Allergen Online database for domain-level similarity testing.

Bioinformatics evaluation of genetically modified proteins expressed in Bt-Brinjal using FASTA program

The FASTA software package allows users to search nucleotide or protein databases using a query sequence. It is available at The European Bioinformatics Institute web server. For a query of the same kind, FASTA itself conducts a local heuristic search of a protein or nucleotide database. The comparison seeks to ascertain whether the protein sequence in question is homologous or identical to any known or suspected allergens.

Results and Discussion

In the present study the translated protein sequences of cry1Ac, cry1Ab, and cry1Fa1 from *Bacillus thuringiensis* were examined to see whether they would be allergenic in Bt brinjal (Table 1).

In silico assessment of the genetically modified proteins expressed in Bt-Brinjal using online databases

To assess for a potential allergic match, *in silico* test was performed to find the homology between genetically engineered proteins and known allergens. Searches were performed using various allergen databases on the complete/partial (80mer/8mer/6mer) sequences of cry1Ac, cry1Ab, and cry1Fa1 synthesized in Bt-Brinjal. None of the known allergens showed a significant match of more than 35% according to Allergen Online version 21.0, AlgPred, or SDAP. For all questioned Cry protein sequences, a maximum percent identity of less than 50% was determined (Table 2). Aalberse (2000) asserts that a protein having greater potential for cross-reactive or IgE-binding provided that it shares more than 70% of its length with an allergen, compared to proteins that share less than 50%. The Codex, 2003 criteria, which stipulates that minimum 35% identity across an 80-amino acid match suspect probable allergenic cross-reactivity of genetically modified proteins that are inserted into a GM crop (Alimentarius,2003), was not met by these results.

In silico evaluation of genetically engineered proteins expressed in Bt-Brinjal using NCBI's BLASTX tool

According to the NCBI BLASTX search results, the proteins encoded by cry1Ac, cry1Ab, and cry1Fa1 had homology with a number of insecticidal crystal proteins from *Bacillus thuringiensis*, but no homology found with the allergenic proteins (Table 3).

Comparison of the allergen and cry protein domains

Using Interproscan, the domains of the novel cry proteins were generated. When the domains of the novel cry protein and the Allergen Online database domains were evaluated, they did not match, eliminating the potential for allergen cross-reactivity even when there is no sequence resemblance.

Bioinformatics evaluation of genetically modified proteins expressed in Bt-Brinjal using FASTA program

Using Pearson's standard search and scoring matrix, a complete FASTA protein similarity search was carried out (Pearson, 2000). Finding out whether the protein sequence of cry1Ac, cry1Ab, or cry1Fa1, is homologous to any known allergens in the database is the goal of the comparison. Table 4 displays the alignment's outcomes. A low degree of homology between the database and query proteins is indicated by a high E score.

The marketing of genetically modified foods must be systematically regulated to confirm that the resulting foods are equally safe for the environment. The study of the safety of new proteins produced through genetic engineering is one aspect of the safety assessment of agricultural commodities. Based on the information of proteins' organic and inorganic characteristics and different test techniques for assessing the inherent risks of chemicals, two-stage system has developed by International Life Science Institute (ILSI) for safety evaluation of genetically modified proteins used in agricultural biotechnology. Tier I considerations include the evaluation of protein's biological function or method of operation and anticipated use, prior safe usage, comparison of genetically engineered protein's amino acid sequence with other proteins, and biochemical and physicochemical qualities (identification of potential hazards). When Tier I data are insufficient to determine an individual's safety (reasonable certainty that no harm will come to them), Tier II (hazard characterization) is used. An increasing number of Bt plants are being introduced for commercial cultivation globally that express single, stacked, or pyramidal Cry proteins. According to the results of the in vitro investigation, Cry1, Cry2, and Cry3 degrade quickly, typically in less than 30 seconds (Betz *et al.*, 2000). These in vitro tests show that consuming Cry proteins causes rapid and extensive protein degradation. Since there is a much lower chance of absorption, the risk for an allergic reaction is minimized by the rapid breakdown of the Cry1, Cry2, and Cry3 protein groups after eating. While unremarkable proteins from common foods of being allergic are swiftly destroyed in simulated gastric fluid, food allergens typically linger in the gastrointestinal model (Astwood & Fuchs, 1996).

Table.1 Sequence information of cry1Ac, cry1Ab and cry1Fa1

Gene Name	Organism Name	GenBank Accession No	UniProtKB/ Genpept ID	Protein Name	Sequence Length (Protein)	InterPro Domain ID
cry1Ac	*Bacillus thuringiensis*	Y09787	O32306	**Crystalineentomocidal protoxin**	618	IPR005369 IPR005368 IPR001178
cry1Ab	*Bacillus thuringiensis*	X54939	P0A372	**Pesticidal crystal protein**	1155	IPR041587 IPR005639 IPR001178 IPR005638
cry1Fa1	*Bacillus thuringiensis* serovar aizawai	M73254	AAA22347	Insecticidal delta endotoxin	1174	IPR041587 IPR005639 IPR005638 IPR001178

Table.2 Sequence homology search of cry1Ac, cry1Ab and cry1Fa1 proteins in allergen databases

Protein Query		Allergen Online version 21.0	Algpred	SDAP
cry 1Ac	**Full sequence**	No identity matches >35% found	Does not contain experimentally proven IgE Epitope	No match found with E-value<0.01
	80 amino acids	No identity matches >35% found	NA	No match found with E-value<0.01
	8 amino acid	No identity matches >35% found	NA	No match found with E-value<0.01
	6 amino acid	NA		NA
cry 1Ab	**Full sequence**	No identity matches >35% found	Does not contain experimentally proven IgE Epitope	No match found with E-value<0.01
	80 amino acids	No identity matches >35% found	NA	No match found with E-value<0.01
	8 amino acid	No identity matches >35% found	NA	No match found with E-value<0.01
	6 amino acid	NA	NA	NA
cry1Fa1	**Full sequence**	No identity matches >35% found	Does not contain experimentally proven IgE Epitope	No match found with E-value<0.01
	80 amino acids	No identity matches >35% found	NA	No match found with E-value<0.01
	8 amino acid	No identity matches >35% found	NA	No match found with E-value<0.01
	6 amino acid	NA	NA	NA

*NA: Not Applicable

Additionally, the diet's quite low concentration of these genetically modified Cry proteins significantly reduces the exposure and, hence, the chance of absorption. The likelihood that a novel gene will encode an allergen is very high. A recognized allergen being transferred from one source to another is one probable allergy concern associated with genetically engineered food crops. An immunological cross-reaction may happen as a result of the proteins' close structural resemblance. The newly introduced protein's degree of similarity to a recognized allergen must therefore be ascertained. To determine whether a protein is safe, its primary amino acid sequence and general structure must be compared to those of known allergens.

Various bioinformatics tools are available for the evaluation of potential allergenicity, and authenticated epitopes can be used in addition to various online databases to analyze each protein for allergenic potential. The current study describes how several bioinformatics tools and approaches were used to assess cry1Ac, cry1Ab, and cry1Fa1 proteins produced in genetically modified brinjal for probable allergic cross-reactivity. AlgPred, SDAP, and Allergen Online version 21.0 results showed no homology with identified allergens. None of the genetically modified proteins meet the requirements for possible allergic cross-reactivity, according to BLASTX, domain analysis, and FASTA searches.

Table.3 BLASTX results for cry genes

Query Protein sequence	Subject/Database sequence	Maximum score	Total score	E-value	Percent identity	Accession
cry1Ac	Cry1AcCry1I [synthetic construct]	1270	1622	0.0	100.00%	AGS47767.1
	Cry1Ac [synthetic construct]	1257	1257	0.0	99.68%	AJU57501.1
	insecticidal delta-endotoxin Cry8Ea1 family protein [*Bacillus thuringiensis*]	1254	1254	0.0	99.03%	WP_243510
cry1Ab	Cry1A-like protein, partial [*Bacillus thuringiensis*]	2378	2378	0.0	97.00%	AEH31425.1
	delta-endotoxin [*Bacillus thuringiensis*]	2377	2377	0.0	99.91%	AFK79795.1
	Cry1Ab, partial [*Bacillus thuringiensis*]	2377	2377	0.0	99.91%	AEV45790.1
Cry1Fa1	insecticidal delta-endotoxin Cry8Ea1 family protein [*Bacillus thuringiensis*]	2406	2406	0.0	100.00%	WP_106001067.1
	Cry1F-like protein, partial [*Bacillus thuringiensis*]	2404	2404	0.0	99.00%	AEH31423.1
	CryINA67-1 [*Bacillus thuringiensis* serovar morrisoni]	2050	200	0.0	99.00%	BAA25298.1

Table.4 FASTA results for cry genes

Query Protein sequence	Database ID	Subject sequence	Length	Score(Bits)	Identities %	E-value
cry1Ac	TR:O32306	Crystaline entomocidal protoxin *[Bacillus thuringiensis]*	618	959.5	100.0	0.0
	TR:E3TBL2	Cry1Ab/c [synthetic construct]	640	959.5	100.0	0.0
	TR:E3TBL1	*Cry1Ac [synthetic construct]*	1178	959.2	100.0	0.0
cry1Ab	TR:Q7BE98	Crystaline entomocidal protoxin *[Bacillus thuringiensis]*	1155	1798.5	100.0	0.0
	SP:P0A370	Cry1Ab [*Bacillus thuringiensis* subsp. Kurstaki]	1155	1798.5	100.0	0.0
	SP:P0A372	*Cry1Ab[Bacillus thuringiensis subsp. Aizawai]*	1155	1798.5	100.0	0.0
Cry1Fa1	SP:Q03746	*Cry1Fa [Bacillus thuringiensis subsp. Aizawai]*	1174	1843.6	100.0	0.0
	TR:V9I164	Crystaline entomocidal protoxin (Fragment) [*Bacillus thuringiensis*]	1174	1842.2	99.9	0.0
	TR:Q45749	Crystaline entomocidal protoxin (Fragment) [*Bacillus thuringiensis*]	1174	1602.8	86.5	0.0

The cry1Ac, cry1Ab, and cry1Fa1 insecticidal toxin proteins, which are expressed in Bt-Brinjal, were examined *in silico* to check for any probable allergic cross-reactivity.

The genetically modified proteins of Bt-Brinjal were not identical to allergenic proteins in whole sequence, 80 amino acid match, or 8 amino acid/6 amino acid match, according to several database searches and similarity searches.

Since the potential cross-reactivity of these recombinant proteins with allergenic proteins was not reached, this result can help farmers, researchers and policy makers to promote the cultivation of Bt-Brinjal as far as biosafety is concerned.

Availability of data and material

The datasets used and/or analyzed during the current study are available in the UniProtKB (The Universal Protein Resource Knowledgebase)

Competing interests

The authors declare that they have no competing interests.

Authors Contribution

Sujit Kumar Das: Research concept and design, collection and/or assembly of data, data analysis and interpretation, writing the article, and critical

revision of the article. Sukanta Kumar Pradhan: Research concept and design, and data analysis and interpretation. Kailash Chandra Samal: Data analysis and interpretation, and critical revision of the article. Nihar Ranjan Singh: Research concept and design. The authors read and approved the final manuscript.

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Abbreviations

CRY proteins: Crystal proteins
Bt: *Bacillus thuringiensis*
GM: Genetic Modified
IgE: Immunoglobulin E
GMPs: Genetically Modified Plants
WHO: World Health Organization
BLASTX: (Basic Local Alignment Search Tool X)
ILSI: International Life Science Institute

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