In silico safety assessment and morphological characterization of transgenic Maruca vitrata resistant cowpea (Vigna unguiculata L. Walp)

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ABSTRACT

Prior to the introduction of Genetically Engineered crops into the market, their safety needs to be thoroughly scrutinized for potential allergenicity and toxicity. The use of omics tool in safety assessment has been recognized as a critical tool for generating evidence of safety or otherwise of GE crops, by the Codex Alimentarius Guidelines of WHO and FAO. The objective of this study was to perform the safety analysis of PBR cowpea. For identification of the trait, lateral flow strip test using cry1Ab strips of lot number 6M1053 was used. PCR analysis was conducted for the amplification of the transgene. In addition, the morphological characteristics of the GE crop was evaluated using a non-transgenic cowpea as a standard. In silico analysis of the transgene product was performed to evaluate its three-dimensional x-ray crystal structure using fifteen Allergen and Toxin database. A total of 8,996,415 sequence alignment was conducted using BLASTP 2.2.27+, FASTA35.04 and BLOSUM62 scoring matrix to identify distant homologs. The cutoff for the e-value and maximum identity score was set at 1.0 and 35% respectively. The toxic or allergenic criteria were not met in all the database used, suggesting that the Cry1Ab transgene of the GM cowpea is safe for consumption. Analysis by sliding 80mer, sliding 8mer and 6 amino acid exact word-match also confirmed the transgene and its source organism are non-allergenic and risk-free. Evaluation of the morphological characteristics of the event also revealed that the transgene did not lead to any phenotypical alteration of the plant.

Keyword: *Transgene, In silico, Cry1Ab, PBR Cowpea, Allergenicity*

1. INTRODUCTION

Safety analysis of Genetically Engineered (GE) crop is one of the key-regulatory factors for their commercialization [1, 2. 3]. Critical elements in risk assessment of transgenic events comprise of allergenicity and toxicity potentials of the GE crop when scrutinizing its safety status [4,5]. Because allergens and toxins affect more than one-third of the world population [6; 7], they are given high priority in any GE crop safety analysis.

The use of in silico omics tools to evaluate the allergenicity and toxicity potentials of novel proteins has seen tremendous progress over the last decade [8] and it is one of the weights of evidence-generating approaches that have been recommended by the Codex Alimentarius Guidelines of World Health Organization (WHO) [9], the European Food Safety Authority, and the US Environmental Protection Agency [10, 11]. While GM and non-GM crops are typically morphologically similar, a standardized demonstration of their morphological characteristics is important for plant breeders and regulatory agencies in order to identify possible changes that may be detrimental to the safety of the environment and to the biology of such plants [5, 12].

Food safety assessment of biotech proteins is performed essentially following the WHO and Food and Agricultural Organization (FAO) guidelines compiled in the Codex Alimentarius document [13] using Tier I approach based on weight of evidence proposed by the International Life Sciences Institute (ILSI). The Tier I (identification of possible hazard) approach encompasses an assessment of the biological function or mode of action, intended application of the protein, History of Safe Use (HOSU), comparison of the amino acid sequence of the protein to other proteins, as well as the biochemical and physicochemical properties of the proteins [14]. The aim of this study was to perform a safety assessment of the cry1Ab event expressed in Maruca vitrata resistant transgenic cowpea, and to carry out the morphological characterization of this plant.

2. MATERIALS AND METHOD

2.1 Seed preparation and collection

Seed preparation and collection

Seeds of transgenic and conventional cowpea were collected from the Institute of Agricultural Research (IAR). Three lines (Line IT86D1010, IT97KT and IT97KN) of cowpea seeds were collected and planted for the extraction of its DNA. The design of the experiment is presented in Figure 1.

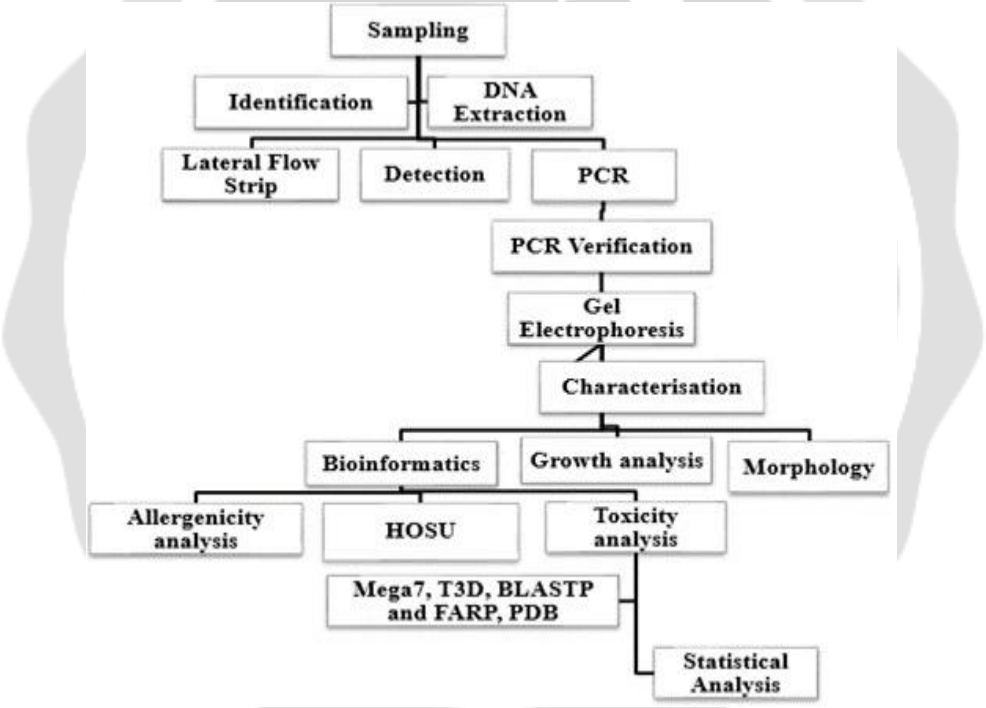

Figure 1: Overview of the experimental design

2.1 Lateral Flow Test

The lateral flow strip test was conducted according to method described by Narjara *et al*. [15]. Collected seeds were grounded and supernatant were obtained after which 0.5 mL was pipetted and added to the reaction tube containing the extraction buffer in which the strip was inserted. The strips were analysed after ten minutes. Transgenic and non-transgenic seeds were used as positive and negative control respectively for each event.

2.2 DNA Isolation

The extraction of DNA samples was done according to Qiagen DNeasy Plant Mini Kit manual (2000) as described by the manufacturer.

2.3 PCR Detection

For assessment of the specificity of the technique in detecting the events, DNA of the plant seeds were extracted and analysed using PCR to detect the transgene in the plant genome. PCR analysis was done using the primer pairs F-5′-GGA TCCATG GAT AAC AAT CCG AAC ATC-‘3; R- 5′-GTC GACTTATTCCTCCATAAGAAGTAA-3′. Bio-Rad PTC-100 Thermal Cycler was used to set the PCR conditions with pre-incubation at 95℃ for 10 min, initial denaturation of 30 seconds at 95℃ and annealing at 59℃ for 30 seconds. The cycles were repeated fifty times. The total volume of the PCR mixture was 25 µL and contained: 50 ng DNA extracted from feed samples (2 µL), 2.5 µL 10 X buffer, 2.5 µL 25 mM MgCl2, dNTPs, primers, 0.1 µL 5 U/µL Taq DNA polymerase and nuclease-free water.

2.3 In silico Analysis of the Potential Toxicity/ Alergenicity of Cry1ab Protein

2.3.1 History of Safe Use (HOSU)

A literature review on the history of safe use (HOSU) of *Cry1Ab* protein was performed according to the principles described by Constable *et al.* [27]. This search was composed of reports for the *Bt* (source of the *Cry1Ab* protein) and three-domain *Cry* proteins [26]. The PubMed and Protal database were accessed using the following combination of keywords: (a) “history of safe use” and “*Bacillus thuringiensis*”; (b) “history of safe use” and “*Cry* proteins” (c) “food/feed safety” and “*Bacillus thuringiensis*” (d) “food/ feed safety” and “*Cry* proteins”, and (e) “risk assessment” and “*Bacillus thuringiensis*”.

2.4 FASTA sequence alignment search for potential toxicity and allergenicity of the Transgenic Cowpea

Fifteen database system including the Pan Pesticide Database (PPD) and the *Toxin and Toxin Target Database* (T3D) were used for the allergenicity and toxicity test respectively. The T3D currently hosts a total of 42,471 toxin and toxin target associations, with 3,673 toxins described by 41,733 synonyms, including pollutants, pesticides, drugs, and food toxins, which are linked to 2,087 corresponding toxin target records [28] while PPD hosts 6,500 pesticides, insecticides and herbicides including toxicity, water pollution, ecological toxicity. FASTA search with a threshold concern of 35% identity as a primary bioinformatics method as highly recommended by Abdul *et al.* [1] was used. Based on the information of scientific literature for sequence identities of clinically demonstrated cross-reactivities, there are very few cross-reactive pairs of proteins that would not be identified by a scanning window of 80 amino acids with a threshold of 35–45% identity [16], to compare against a well-founded allergen database. The sequence alignment search for this study was carried out using scoring matrix of BLOSUM62 with FASTA3.45 and BLATP 2.2.27 algorithm.

2.5 Sliding 80mer/ windows search

Sliding 80mer search was performed using fifteen database. To be consistent with Codex Alimentarius Guidelines of FAO/WHO [13], the calculation of the cut-off value for a match was changed to >35% while the e-value cutoff for the sliding 80mer search was changed from 100 to 10.

2.6 Sliding 8mer/ 6mer windows search

Sliding 8mer/6mer search was done using the Alermatch, SDAP and AllergenOnline database while the Cry protein three-domain (C3-D) *in silico* safety assessment was also done to evaluate the presence of domains similar to those of allergens and toxins. The Interproscan (<http://www.ebi.ac.uk/Tools/ InterProScan/>) was used to deduce the domains present.

2.7 Alignment Algorithms

BLASTP 2.2.27+ was used for comparisons and BLOSUM62 for scoring matrix. Cry1Ab sequences were subjected to FASTA comparisons using as filter e-value cutoff of 1.0 for detection of identity >50% for the complete sequence similarity and >35% in a window of 80 amino acids. These criteria were particularly determined for this work and were based on the study of Moran *et al.* [17].

2.8 Judgment of Result: Full FASTA Sequence Alignment/ Search

Alignments with high identity scores may indicate a potential for allergenic cross-reactions. If a protein shares greater than 70% identity over its length, relative to allergen, it is likely to be cross-reactive and if it has less than 50% identity, it is not very likely to be cross-reactive [18]. The extent of similarity was evaluated by the Maximum Identity Score (MIS) and expectation score (e-value). e-value much smaller than 1 (e.g., 1e-25) indicates a highly

significant alignment, probable evolutionary relationship and, most importantly, a high degree of structural similarity [19; 20].

2.9 Morphological characterization: Line IT86D1010, IT97KT and IT97KN

The plant morphology was carefully characterized using the following parameters: plant growth rate, plant weight, seed (size, shape, colour and appearance), leaves (colour, size, shape, number and appearance) and stem girth.

3. RESULTS AND DISCUSSION

3.1 Detection/Identification of the transgenic event present in the transgenic cowpea

The Flow Strip Event Detection

The flow strip analysis result gave a positive test line for the presence of the Cry1Ab gene in the transgenic cowpea. No positive test line was observed for the other traits. Therefore, this confirms the presence of the Cry1Ab trait in the transgenic cowpea (Plate 1).

Plate 1: Flow strips for *Bt* traits showing positive results for *maruca* resistant transgenic cowpea and negative for the conventional cowpea sample.

PCR Event Identification

The electrophoregram of the PCR products gave a visible band in the IT97KT lane. No visible band was observed in either the lanes of IT86D1010 or IT97KN. This result further confirms the presence of the *Cry1Aab* gene in the transgenic IT97KT and its absence in the non-transgenic cowpea line (**plate 2**).

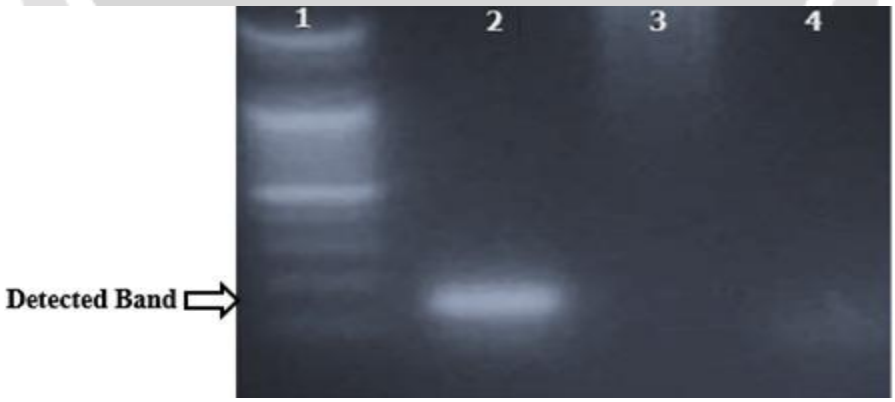

Plate 2: Gel electrophoregram of PCR products of *cry1Ab* gene from cowpea DNA sample. Lane 1, bp marker; lane 2, transgenic cowpea; lane 3, ddH_2O; lane 4, conventional cowpea.

3.2 Generic Sequence Alignment Search for the Allergenicity, Toxicity and Antinutritional Potential of the Cry1Ab gene

The BLASTP search results revealed that the protein coded by Cry1Ab gene showed no homology with any allergens, toxins and antinutrients in the following NCBI Entrez: Non-redundant protein sequences nr; Refrence

protein (ref_seq) database; UniprotKB/Swiss-prot database; Protein data bank protein (pdb) database; and Transcriptome Shotgun Assembly protein (tsa_nr) data base (**Table 1**).

The judgement of this result is based on the fact that proteins that share over 35–70% identity throughout their sequence with an allergen is likely to show cross reaction or to share the same epitopes for IgE [18]. There was an acute decreased in the number of hits (to zero) when the keywords: "toxins", "allergens" and "antinutrients" were entered (with e-score > 1.0 and identity far >35% for the complete sequence).

In silico Toxicity Assessment of Cry1Ab gene

The seven factors-based toxicity searches using the Pan Pesticide Database (PPD) did not identify any similarity or identity with any of the known toxins (**Table 1**).

Table 1: PPD Toxicity information for *Bacillus thuringiensis* Cry1Ab protein

Factors	Result/ Analysis	Remark
PAN Bad Actor Chemical[1]	NL	Not Toxic
Acute Toxicity	NWE	Not Toxic
Carcinogen	NWE	Not Toxic
Cholinesterase Inhibitor	NL	Not Toxic
Ground Water Contaminant	NWE	Not Toxic
Developmental or Reproductive Toxin	NWE	Not Toxic
Endocrine Disruptor	NWE	Not Toxic

NW: No weight of Evidence; NL: Not Listed

Further assessment using the analytical Omics tools of the Toxin and Toxin Target Database (T3D) showed no cross-reactivity, similarity, or identity with any of the toxins registered in T3D (**Table 2**).

Table 2: T3d Database result summary output

Unique Identifications						
Allergen	**Size**	**Bit score**	**Gene bank**	**E cut-off**	**E score obtained**	**Inference**
GLUT-2	524	28.1054	AAA59514.1	1.00	6.42	NT

NT: Not Toxic

3.3 Transgenic cowpea event full sequence Allergenicity Assessment

The full FASTA alignment between the query sequence (Cry1Ab protein) and all the allergies in the database highlighted in table (3) below revealed a MIS much lower than 35%, indicating a low or no degree of similarity between the query sequence and sequence of allergies in the database. The e-value for the query protein was also far higher than the cut-off. Further sequence search in the AllFam database also gave no hit (**Table 3**).

Table 3: Full FASTA Sequence Alignment based on FAO/WHO Allergenicity rules

Database	Allergen	GenBank/ Uniprot	MIS (%)	E-value	Cut off	BS	NIA	Inference
FARRP[a]	*Car b 1 PA*	ABZ81044.1	26.5	1.5	<0.02	-	-	NLA
FARRP[b]	*Major allergen*	CAA50328.1	27.6	2.0	<0.02	-	-	NLA
Allerbase[a]	*Ligv1_ligvu*	O82015	-	2.7	<0.01	26.6	-	CNM
Allerbase[c]	*Pert_human*	P07202	-	9.6	<0.01	25.8	-	CNM
ADFS	*MPA Lig V 1*	KHG25921.1	-	22	<0.02	-	-	AWN
Allermatch[a]	*CYN d 23.0101*	AAP80170.1	-	19.5	<0.02	-	-	AWN
Allermatch[b]	*COR a 1.0102*	CAA50327.1	-	27.6	<0.02	-	-	AWN
SDAP[a]	Tria a gladin	AAA34285	3.62	-	-		36/995	CNM
SDAP[b]	Mala s 1	Q01940	4.12		-		41/995	CNM

[a,b,c:] Different output; CNM: Criteria Not Met; MIS: Maximum Identity Score; NLA: No Likelihood of Allergenicity; NLA: No Likelihood of Allergenicity; PA: Pollen Allergen; BS: Bit Score; AWN: Allergenicity Weight of evidence Not found; No of identical aa: NIA; MP: Major Pollen allergen.

3.3.1 Sliding 80mer windows search for potential allergenicity of the Cry1Ab protein

The Codex Alimentarius criteria [13] requires that potential allergenic cross-reactivity for recombinant protein introduced into a GE crop show at least 35% sequence identity with an 80 amino acid segment. The identity results of the alignments of every possible 80 amino acid segment with the Cry1Ab protein showed that there were no matches of greater than 35% sequence identity (**Table 4**). The highest number of identical amino acid in every 80 count was 22 with MIS of 27.5% and was found at 80 amino acid range of 415-494 which corresponded to Hevb1 allergen (ACN: P15252).

Table 4: Sequence identity search of Cry1Ab protein in SDAP FASTA alignments for an 80 amino acids sliding window using FASTA 3.45 Algorithm

Data Base	Allergen	ACN (GB/uniprot)	AA Match Range	IA 80 Count	MI (%)	MI Cut-Off	Inference
SDAP[c]	Ligv1	O82015	61 - 140	20	25.0	>35%	CNM
SDAP[i]	Musa2.0101	O8VXF1	317 - 396	21	26.25	>35%	CNM
SDAP[k]	Hevb1	P15252	415 - 494	22	27.5	>35%	CNM

IA80 count: Identical Amino Acid in every 80 Count; AA: Amino acid; CNM: Criteria Not Met

Further search using the AllergenOnline and Allermatch database did not didn't identify any hit (**table 5**). This result shows that *Cry1Ab* protein does not meet any of the criteria to be called an allergen.

Table 5: Sliding 80mer windows search

Data Base	No of Hits	No of 80mers	Cut off	Result	Inference
AllergenOnline	0	1076	>35%	NMF	NAA
Allermatch	0	1076	35%	NMF	NAA

Nmf: No Matches Found; NAA: Not an Allergen

3.3.2 8mer Exact Match

Further search using 8mer exact match confirmed cry protein to be not a member of the allergen family (**Table 6**).

Table 6: 8mer exact match result

Data base	No of 8mers	Cut off	Result	Inference
AllergenOnline	1148	>35%	No Sequence found	Not an allergen
Allermatch	1148	35%	No Sequence found	Not an allergen

3.3.3 6 Amino Acid Exact Match of the query protein (Cry1Ab) with known allergen sequence

The highest 6 amino acid exact match obtained is 1 (**Table 7**), giving a percentage identity of 0.09% as against the 35% cut-off set by WHO. The result showed that Cry1Ab protein does not meet any of the criteria to be tagged an allergen.

Table 7: Six amino acid exact word match search

Database	Allergen	NWM	% EWM Obtained	% Cut off	Inference
WHO-IUIS	wi_Tri_a_34	1	0.09	35%	NA
UniProt/ WI	al_Phl_p_11	1	0.09	35%	NA
SDAP	Blot1.0201	1	0.9	35%	NA

NA: Not Allergenic; ID: Identity; %EWM: percentage exact word match; WI: WHO-IUIS

Further search using the structural database 6 Amino Acid Exact Match identity search of the Cry1Ab protein also confirmed Cry1Ab to have no cross reactivity (**Table 8**). The Codex Alimentarius Criteria of WHO [13] requires

that for a protein to show potential allergenic cross-reactivity, there must be at least 35% 6-amino acid exact match between the Query protein (in this case, Cry1Ab) and any of the allergenic or toxic protein. This criterion was not met.

Table 8: Structural Database 6 amino acid exact word match

Database	Allergen	Accession	N of 6 AA word match	MIS (%)	Cut off (%)	Inference
SDAP	Blot1.0201	AAQ24541	1	0.9	35	CNM
SDAP	PhAA1	Q41260	1	0.9	35	CNM
SDAP	CupA1	Q9SCG9	1	0.9	35	CNM

MIS: Maximum Identity Score; CNM: Criteria for Allergenicity Not Met; N: Number; AA: Amino Acid

3.4 Cry Protein Three Domain (C3_D) *In silico* Toxicity and Allergenicity Assessment Using Interproscan

The C3_D *in silico* analysis of cry protein performed using the Interproscan omics tool did not reveal any match between the Cry protein domains and the allergen domains during the domain alignment. The galactose binding domain like (orange bar) (Figure 2a, b and c) was also not identified with any toxins or allergens repeat.

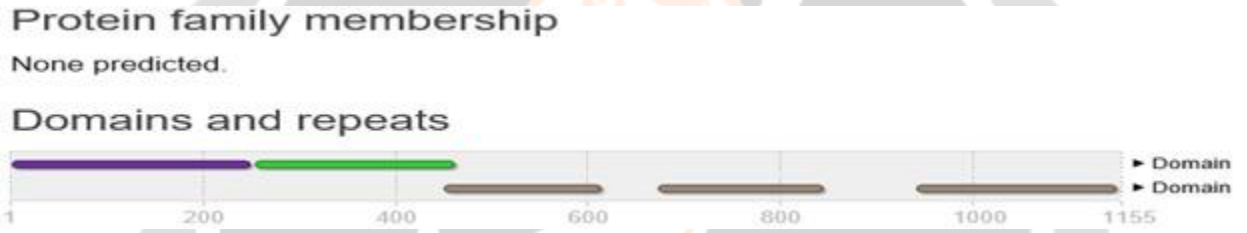

Figure 2a: Domains and repeats

Figure 2b: Detailed signature matches

Figure 2c: Interproscan output result search: No matches were found between the cry protein and any of the speculated domain

3.5 History of Safe Use (HOSU)

More than 1,800 papers (from 1959 to 2017) were reviewed for this study. The HOSU of cry protein three domain (c3_d) was performed using PubMed and Protal database following the principles described by Constable *et al.* [27]. This search reveals that the protein has a long history of safety as summarized in table 9.

Table 9: History of Safe Use (HOSU) of cry proteins using the PubMed and Portal Database

Cry proteins	Result	Findings	Reference
Cry1Ab	NSD	*Cry1Ab* proteins are safe for the lacewing, *C. pallens*	Ali *et al.*, 2017
Cry1Ac/2Ab	NSD	No detrimental effects on adult honeybee	Niu *et al.*, 2017
Cry1Ac/2Ab	CD	Negligible exposure of phloem sucking hemipterans	Meissle, 2017
Cry1Ab/2Aj	r_m	No lethal or sublethal effects	Zhang *et al.*,2017
Cry1Ac	CLT	No adverse effect on NTO	Wang *et al.*, 2017
Cry1Ab	NSD	Stable aphid population density in *Bt* rice fields.	Ren*et al.*, 2016
1Ab/1ac	RB	Rapid degradation of the cry protein found.	Liu *et al.*, 2016
1Ab/1Ac	NSD	GM rice is equivalent to its parental rice line MH63	Mao *et al.*, 2016
Cry1Ie	NSD	No adverse effect on midgut bacteria diversity	Jia *et al.*, 2016
Cry1Ac	HLE	High dose criterion met up to 50 times.	Dourado *et al.*, 2016
Cry1Ab/1Ac	NSD	Frog development was not affected by dietary intake	Zhu *et al.*, 2015
Cry1Ab/1Ac	NSD	Safe for use as feed and food	Li *et al.*, 2015
Cry1C/2A	NSD	No acute toxicity to A. mellifera (honey bee) larvae.	Wang *et al.*, 2015
Cry1Ac	RB	No Cry protein was detected in the soils surrounding	Xiao *et al.*, 2015
Cry1Ac	NSD	No adverse effects in RS of male rats	Guo *et al.*, 2015
Cry2Aa	NSD	No detrimental effects on C. lividipennis	Han *et al.*, 2014
Cry1Ah	NSD	No adverse immunotoxicological effects of GM corn	Song *et al.*, 2014
Cry1Ab	NSD	No significant long-term (90 day) toxic effects	Wang *et al.*, 2013
Cry1Ab	NSD	No significant long-term (90 day) toxic effects	Wang *et al.*, 2013
Cry1Ab	NSD	No significant long-term effects on female Swiss rat.	Wang *et al.*, 2013
Cry1Ac-M	NSD	BT-38 maize is as safe as its conventional maize.	Liu *et al.*, 2012
Cry3Bb1	NSD	No adverse effects on various NTO	Devos *et al.*, 2012
Cry1Ab/Ac	NSD	Safe for use as feed and food	Xu *et al.*, 2009
Cry1ab	NSD	No potential risk of transfer	Guertler *et al.*, 2009
Cry1Ab	NSD	No toxicity was observed even at high concentrations	Bondzio *et al.*, 2008
Cry1Ab	NSD	No adverse effects of *Cry1Ab* in the 90-day study	Schrøder *et al.*,2007
Cry1Ac	NSD	GM grains were equivalent to their non-transgenic	Li *et al.*, 2007
1Ac/2Ab2	NSD	GM cotton is safe for food and use	Hamilton *et al.*2004
Cry1Ab	AS	*Bt* hybrids can increase the percentage of corn grain that would be suitable for use in food and feed	Hammond *et al.*, 2004
Cry1Ac	AS	No clinical abnormalities observed	Spenser *et al.*, 1996
Cry1Ab	AS	No toxicity was observed	Noteborn *et al.*1994
Cry1Ac/3A Cry3Ba	AS	No evidence of toxicity or infectivity/pathogenicity.	Carter *et al.*, 1993
1Aa/1Ab	AS	No evidence of toxicity/ infectivity/ pathogenicity.	David, 1989
Cry1Aa/1Ab/1Ac/2A	NSD	Not a virulent or invasive mammalian pathogen.	Hadley *et al.*, 1987
Cry1Aa/1Ab/1Ac/A	NSD	No toxic or virulent effects found	EPA Fact Sheet, 1986
Cry1Ab Cry1B	NSD	All subjects remained well throughout the 5 weeks experiment	Fisher and Rosner, 1959

NS: Not Specified; RS: Reproductive System; NSD: No significant difference; AS: Acceptable Standard; HLE: High Level of Efficacy; CLT: Consistent Life-Table parameters; CD: Complete Digestion; RB: Rapidly biodegradable; NTO: Non-Target Organism; MCC: Meiobenthos Community Composition.

3.6 Morphological Characterization of the three cowpea lines: Line IT97KT, IT86D1010 (Transgenic), and Line IT97KN (Non-transgenic)

As shown in **plate 3**, no differences in appearance exists between the transgenic cowpeas (IT86D1010 and IT 97KT) and the non-transgenic cowpea (IT97KN). The Plant types are erect. Growth habits were observed to be fairly determinate. The plant was strongly tap rooted. Seeds developed a kidney shape when not restricted within the pod. When seed growth was restricted by the pod the seed becomes progressively more globular (**Plate 3**).

Plate 3: Seeds of three different lines of cowpea. Line IT86D1010 and IT97KT are transgenic while line IT97KN is non-transgenic

3.6.1 Crop Growth Analysis

The transgenic cowpea seedlings had better growth characteristics such as greener leaves, higher number of leaves, bigger leaves, bigger stems, leaf area, shoot height and greater canopy than the conventional cowpea seedlings (**Plate 4**). During the growth periods, the seed coat was observed to be smooth and green for all the three lines: IT86D1010, IT97KT and IT97KN lines. At germination, emergence was epigeal for all the three lines (as it is in common bean and lupin). There was an alternate trifoliolate leaf development in the IT86D1010, IT97KT and IT97KN lines. During the period of flowering, flowers were borne in multiple racemes on 8 to 20 in. flower stalks (peduncles) that arise from the leaf axil. Three pods per peduncle were observed. The open display of flowers above the foliage and the presence of floral nectaries were also peculiar to the three lines. Plate 4 shows the picture of the three lines at 40 days after planting (DAP).

Plate 4: Leafs of three different lines of cowpea. Line IT86D1010 and IT97KT are transgenic while line IT97KN is non-transgenic. *ALP: Affected Leaf Plant; DAP: Days After Planting*

Plant Height

There was significant variation in plant height among the three cowpea lines. Line graph analysis showed a consistent increase in plant height from day 10 to 30 DAP. However, no increase in height was observed at 35 and 40 DAP (**Figure 3**). Line IT86D1010 was consistently the tallest followed by Line IT97KT and IT97KN throughout the sampling periods (Figure 3). This result is in conformity to the results observed by Nkaa *et al.* (2014).

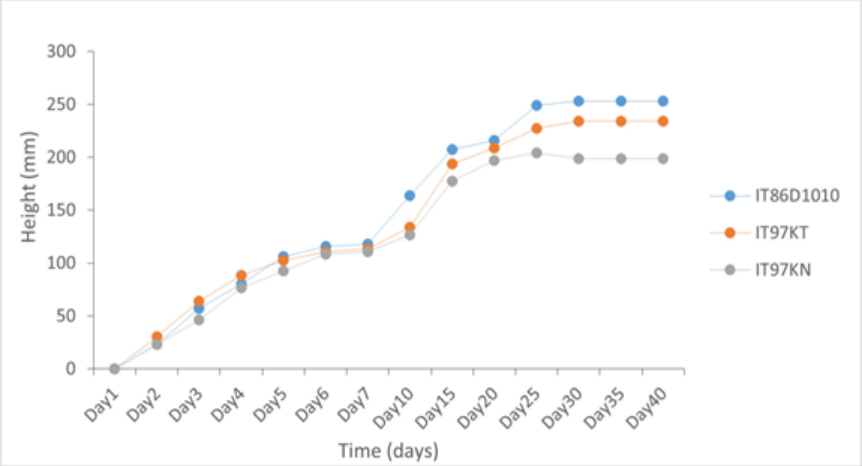

Figure 3: Line graph showing the growth rate by height of the three lines of Cowpea: IT86D1010, IT97KT: Transgenic cowpea and IT97KN: Conventional cowpea

Plant Weight

The plant weight was significantly different between the three crop lines at 10 DAP ($p \leq 0.005$). Line IT86D1010 had the highest weight (5.1g) followed by line IT97KT (2.9667g), while line IT97KN has the lowest weight (2.2667g) at 10 DAP. No significant difference exists at 20, 30and 40 DAP between the three crop lines (T**able 10**).

Table 10: Growth Weight

Crop lines	Crop Growth Rate by Weight (gG^2 $dayG^1$)			
	10 DAP	**20 DAP**	**30 DAP**	**40 DAP**
IT86D1010	5.1000±0.1[a]	11.6000±0.2[a]	9.7333±2.8095[a]	12.9333±4.20991[a]
IT97KT	2.9667±0.05774[b]	8.8000±0.1[a]	9.5667±0.4042[a]	13.7667±0.3055[a]
IT97KN	2.2667±0.05774[c]	26.4000±34.728[a]	7.2333±0.586[a]	9.2000±0.300[a]

N=3; ***abc****Values within the treatment group in the same column followed by same superscript (s) are not significantly different at ($p \leq 0.05$) according to DMRT; DAP: Days After Planting; ±: SD; CGRW: Crop Growth Rate by Weight*

The line graph representations of this data show the trend in the weight variation of these lines (**Figure 4**).

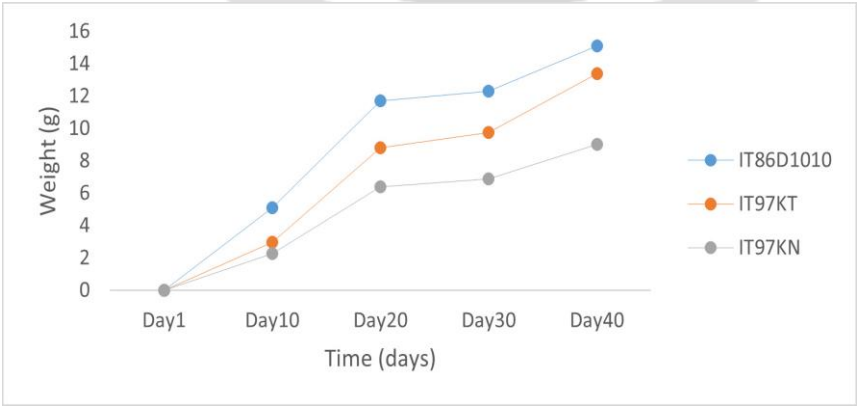

Figure 4: Line graph showing the growth rate by weight of the three lines of Cowpea:

IT86D1010: Transgenic cowpea, IT97KT: Transgenic cowpea and IT97KN: Conventional cowpea

The growth for the three lines were calculated using the Radford (1967) formula. The growth rate for IT86D1010 was 0.24 (g m^{-2} day^{-1}), IT97KT, 0.23 (g m^{-2} day^{-1}) and IT97KN was 0.22 (g m^{-2} day^{-1}) (**Figure 5**).

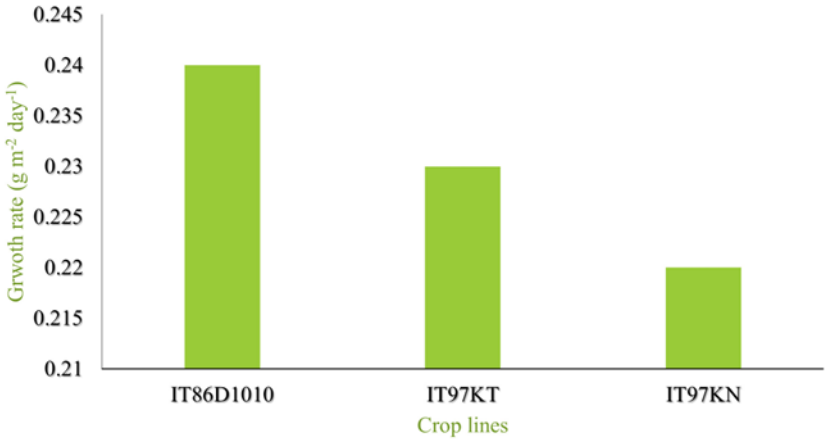

Figure 5: Growth rate comparison of three different cowpea lines. Line IT86D1010 and IT97KT are transgenic while line IT97KN is non-transgenic

Discussion

The need for the safety analysis of transgenic cowpea is predicated on the recommendation of WHO (Abdul *et al.,* [1] as the *in silico* safety analysis of this crop as prescribed by Najaf *et al.* [21]. In the early study of Stadler and Stadler [22], bioinformatics tools were also strongly prescribed and employed. Similar studies carried out in Iran by Najaf *et al.* [21] on allergenicity and toxicity assessment of novel GE foods also confirmed *Cry1Ab* protein safe and nontoxic. Fred *et al.* [23] described rapid in vitro digestibility of *Cry1*, *Cry2*, and *Cry3* classes of proteins using simulated gastric fluids. Results of seven in vitro assays conducted with representative *Cry1*, *Cry2*, and *Cry3* proteins established that the proteins are rapidly degraded, usually within 30s [23]. Morphological characterization of the three cowpea lines also revealed that no difference exists in the seed shape, colour, size and texture and is in agreement with similar studies carried out by Mohamed *et al*. [12] where transgenic and non-transgenic cowpea line expressing *Cry1Ab* gene showed no significant differences in all the phenotypical parameters compared. The non-alteration of the characters other than for which transformation was done was highly desirable in the earlier studies of Khan [24]. The differences in plant height could be attributed to genetic effect of individual varieties.

One of the important considerations in assessing any GE crop is the possibility that the newly introduced gene may encode an allergen or toxin. Since proteins that are structurally very similar may be immunologically cross-reactive, it is also important to determine whether the newly introduced protein is significantly similar to any of the known allergen(s) or toxin(s). The windows search of 80, 8 and 6 amino acids with identity greater than 35% between the query sequence and the subject sequence also showed no similarities. The same results were observed for similar studies carried out on Cry1C and Cry1Ab/Ac proteins by Cao *et al.* [25].

As part of the main objectives, the current study conducted a substantial equivalence assessment of the transgenic Maruca vitrata-resistant cowpea (Vigna unguiculata L. Walp). Substantial equivalence is typically determined through visual observations and basic morphological assessments, as established by regulatory guidelines. While this approach is effective for initial evaluations, it has its limitations, especially in capturing detailed structural and compositional differences that may exist between transgenic and non-transgenic plants.

We acknowledge the suggestion to include advanced morphological analysis techniques such as optical microscopy or scanning electron microscopy (SEM) to provide a more detailed characterization of the cowpea. These techniques could reveal microstructural differences and provide insights into the cellular and tissue-level impacts of the genetic modification. However, these methods were beyond the scope of the current study, which focused on traditional, regulatory-compliant assessment methods.

Our findings demonstrate that the transgenic cowpea is substantially equivalent to its non-transgenic counterpart based on naked-eye observations and basic morphological parameters. The observed traits included plant height, leaf shape, pod morphology, and seed characteristics, which showed no significant differences between the transgenic and non-transgenic plants.

Despite these promising results, we recognize that further studies incorporating advanced imaging techniques could strengthen the understanding of the transgenic cowpea's morphological attributes. Future research should include optical microscopy and SEM to provide a more comprehensive morphological characterization. This would not only enhance the robustness of the safety assessment but also address any subtle changes that might not be visible to the naked eye.

4. CONCLUSION

The results from the omics tools of 16 databases employed in the present study indicates that none of the Cry proteins were found positive for potentially allergenic cross-reactivity and toxicity. Hence, the criteria for potential cross-reactivity have not been reached. Furthermore, the three domain Cry proteins search also revealed no similarities at the domain level. Hence, the bioinformatics search results indicate no need for further in vitro testing such as serum IgE-binding studies. Further comparisons made between the transgenic and non-transgenic cowpea lines also showed no significant differences in all the parameters compared connoting that the transgene has not caused any alteration in the biology of the plant other than that which it was intended for. Base on the results from this study, it can therefore be concluded that Nigeria's transgenic PBR cowpea is safe and good for consumption. The data generated in this study confirms the safety of the GE cowpea.

While our study provides a solid foundation for the safety and substantial equivalence of the transgenic Maruca vitrata-resistant cowpea, integrating advanced morphological analysis in future investigations will offer a deeper and more precise evaluation, ultimately contributing to the broader acceptance and regulatory approval of genetically modified crops.

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