IDENTIFICATION AND CHARACTERIZATION OF POSSIBLE GENETICALLY MODIFIED MAIZE (*Zea mays*), SOYBEAN (*Glycine max*) AND *IN SILICO* ANALYSIS OF TRANSGENIC COWPEA IN NORTHERN NIGERIA

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER DEGREE IN BIOCHEMISTRY

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SEPTEMBER, 2018

DECLARATION

I hereby declare that the work in this dissertation entitled **"Identification and Characterization of possible Genetically Modified Maize (*Zea mays*), Soybean (*Glycine max*) and *in silico* analysis of transgenic Cowpea in Northern Nigeria"** was carried out by me under the supervision of Dr Abdulrazak Baba Ibrahim and Professor Kola Mathew Anigo. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this dissertation was previously presented for another degree or diploma at any other institution.

_____	_____	_____
Name	Signature	Date

CERTIFICATION

This dissertation entitled **"Identification and Characterization of possible Genetically Modified Maize (*Zea mays*), Soybean (*Glycine max*) and *in silico* analysis of transgenic Cowpea in Northern Nigeria"** by Isah, Abraham, meets the regulations governing the award of the degree of Masters of Science (MSc.) of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to God Almighty and to my beloved nation, Nigeria.

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ABSTRACTS

The commercial release of Genetically Modified (GM) grains expressing resistance against the herbicide *imazapyr*, in some countries has led to improved agricultural outputs. Despite the high concern of the possible existence of GM crops in Nigeria, no comprehensive survey has been made to track the possible availability of such events. The aim of this study is to establish a biosafety database of GM Crop under confined field trial and those from possibly available or illegally introduced GM crops in northern Nigeria. Seeds of soybean and maize were randomly collected from major markets across Northern Nigeria. Lateral flow strip test was conducted using *Bt1Ac* test strips of lot number 6M1053, *Cry1Ac* test strip of lot number 6G1027, LibertyLink *PAT* strips of lot number 030023 and *Cry2A* test strips of lot number 031157. Morphological characterization was done using the seeds and leaves of the plant. The transgenic event was characterized using a range of technique including Polymerase Chain Reaction (PCR) and 16 allergen and toxin database. No transgenic event was detected in any of the commercialized maize and soybean samples. PCR analysis using *PAT, Cry* and *CP4 SPSPS* primers also confirmed the absence of any of the respective transgenic event. Conclusively, no transgenic event was found in the grains tested across the various markets of Northern Nigeria based on the samples analysed in this study. The 8,996,415 sequences alignment conducted using $BLAST_P$ 2.2.27+, FASTA35.04 and BLOSUM62 scoring matrix with an e-value cutoff of 0.1 and Maximum identity score of 35%, did not meet any of the toxin or allergen criteria; implying that the *Cry1Ab* gene introduced in the transgenic cowpea is safe for consumption. The Sliding 80mer, Sliding 8mer and 6mer exact word match conducted also confirmed the transgene and its source organism to be safe, non-allergenic and risk-free to humans.

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ABBREVIATIONS, DEFINITIONS AND GLOSSARY

AAD-12……Aryloxyalkanoate di-oxygenase 12 protein

amy797E……A chimeric thermotolerant alpha-amylase *gene*

avhppd-03……..…P-hydroxyphenylpyruvate dioxygenase

bar…….……..…A gene which confers resistance to the herbicide

BBX32…..……An Arabidopsis B-Box Protein bialaphos

bla…….……..Beta lactamase enzyme

BMA……British Medical Association

Bt……*Bacillius thurigiensis*

CFT……..……Confined Field Trial

cordapA……..Gene that codes for dihydrodipicolinate synthase

CP4…….…...*Agrobacterium tumefaciens* strain

Crispr...……...…..…Clustered Regularly Interspaced Short

Cry1Ab……..……Pesticidal crystal protein

DNA……...…….……Deoxyribonucleic Acid

ERA……Environmental Right Association

FATB1…….……Fatty acyl-ACP thioesterase B gene

GMO……Genetically Modified Organisms

HOSU……...……History of Safe Use

hppdPF W36…….……Modified p-hydroxyphenylpyruvate

hra……Heat-resistant agglutinin (*hra*) *gene* fragment

in silico…..……..In computer simulation

mEPSP……..……5-enolpyruvylshikimate-3-phosphate modified

ms45…..……..……..…Homozygous recessive male sterile construct

Nc.Fad3…..…….*Neurospora crassa.* delta-6 desaturase gene

PAT…….……..……..Phosphinothricin acetyl transferase gene

PCR…….……..……..Polymerase Chain Reaction

Pj.D6D……..……..*Primula juliae* delta 6 desaturase gene

PMI…….Phosphomannose isomerase gene

rDNA……...……..…….recombinant DNA

RUR HS……..……Roundup Ready Herbicides Resistance Soybean

TALEN……...…….……Transcription activator-like effector nucleases

uidA……..…….……Beta-glucuronidase gene

vip3Aa20……..…Vegetative insecticidal protein (vip3Aa variant)

zm-aa1……Alpha amylase enzyme gene

zm-hra……..…Herbicide tolerant acetolactase synthase gene

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Genetically modified organisms (GMOs) also called transgenic organisms (Smita, 2015), are living organisms whose genome have been manipulated in a way that would not happen in nature (Patrick and Alan, 2005). Genetic modification or engineering is simply the removing, modifying, or adding genes to a DNA molecule of an organism in order to change the information it contains (Gidado, 2016). The application of genetic engineering to crop breeding and food production is an extension of traditional agricultural technology (Gideon, 2002). Around the world, the predominant genetically modified (GM) crops include soybean, maize and cotton (Pellegrino, 2018). Less common are GE plants with traits that are expected to influence virus resistance, crop quality, male sterility and disease resistance. In addition to the recommendation by Yasin and Mulugeta (2015) that agricultural biotechnology should be used as a powerful tool to complement conventional methods in meeting food demands worldwide, several proofs exist that genetic engineering including RNA interference (RNAi) technique (Ibrahim and Aragão, 2015) has the potential to revolutionize food production (Hammer, 2003). The four main approaches by which plants are manipulated by genetic engineering include: Gene insertion using a bacterial vector; Micro ballistic impregnation; Poration and Gene neutralization by means of antisense technology, homologous recombination (gene knockout) and gene replacement (Gideon, 2002). Despite the potential benefits of such techniques, serious concerns have been raised about the potential environmental and medical consequences of GMOs. Such concern can be clarified by a priori risk assessment for GM crops (NRC 2002; Sharples, 1991). A fundamental way of carrying out a priori risk assessment for GM crops is by determining and

identifying the extent of sequence similarities between protein(s) expressed in such crop and database sequences of allergens/toxins (Gurinder *et al.,* 2011). The British Medical Association (1999), published a report on GMO that addressed three key areas of potential health effects: Transfer of antibiotic resistance, toxicity and allergenicity. The importance of further research on the potential allergenicity and toxicity of GM products was also underscored in this report. Braun (2002) stated that pharmaceuticals and vaccines made by genetic engineering are well accepted all over the world, however, there are many people who are worried that food made by the same technology, may harm their health or cause damage to the environment. Prior to the introduction of the GM crops into the market, their safety need to be thoroughly scrutinized for their potential allergenicity and toxicity (Gurinder *et al.,* 2011). A 'weight of evidence' approach including bioinformatics is recommended by the Codex Alimentarius Guidelines to assess the risk of allergenicity of GM crops and products. Am ong the various strategies employed for asse-ssing the potential allergenicity, a prerequisite is *in silico* sequence analysis (Stadler and Stadler, 2003).

1.2 Statement of Research Problem

The human population will reach ten billion within coming decades (2050-2060), and this calls for urgent concern as already six children die of starvation per minute with one in every nine people going to bed hungry each night (Mercy-Corps, 2018). Already in Nigeria, South Sudan, Somalia and Yemen, there are twenty million people at risk of famine (Mercy-Corps, 2018). To keep pace with population growth, it is estimated that the world will have to produce 50% to 100% more food than at present (Cohen and Paarlberg, 2004) and conventional plant breeding alone will not

be able to sustain the rapidly rising global food demand. Global food production has continually been threatened by a large number of pathogens and pests (Ibrahim and Aragão, 2015), environmental degradation (Pamela, 2011), and climate change (Lobell *et al.,* 2008). Already in developing countries, up to one-third of the food produced around the world is never consumed due to inadequate food production systems (Mercy-Corps, 2018) and thereby results to food wastage. Though the application of agricultural biotechnology has the potential to revolutionize food production (Hammer, 2003), concerns of biotechnology have put considerable constraints on

the opportunity for extended commercial use in Africa and has cost thousands of lives (Arthur, 2011). Example of such concerns include fears about transgenic products as biological weapons (Dando, 2011) and their possible effects of alimentation on the human body (Marta *et al.,* 2013). In Africa, including Nigeria, many governments are skeptical of GM foods (Eicher *et al.,* 2006) despite the suggestions by Asante (2008) that African governments can regulate the acceptance and adoption of GM food and related technologies, by commissioning experts to critically analyze

and evaluate the safety of the technology on human health. Genetic engineering is one of the most contested technologies ever introduced (Gidado, 2016). The organic farming movements have spread fears concerning the use of genetically modified foods and as a result have hindered the potential of GM crops anywhere in the world. In line with these, the present study tend to ascertain the possible existence of GM crops in the northern region of Nigerian markets and the safety status of the GM cowpea under Confined Field Trial (CFT).

1.3 Justification

The reason for the discrepancy between public opinion and scientific evidence needs scientific clarification. The fear about the presence of GM crops in Nigeria has accrued among stakeholders and individuals. One of the potential channels of GM organisms in the country is food aid. Nigeria received 11,000.6 metric tons of soy meal as food aid from the US Food for Progress program in 2003. Taking into account that over 80% of soy beans in the US are GM, it is therefore likely that Nigeria has always received GM Foods. Controversies that have been generated over the adoption of GM foods are numerous, and thus, it calls for an urgent scientific appraisal. Many Sub Saharan African leaders have been reluctant to approve green biotechnology due to widely circulated fears, insufficient knowledge, and civil strife. For example, Namibia cut off all corn trade with South Africa in 2004 because the latter grew GM crops.

In Nigeria, while several claims exists that there are GM crops in the market, others have declared it absent. The need to therefore inform the public about the current status of GM crops in Nigeria via scientific proofs is crucial and expedient. Considering the high porosity of Nigeria borders and the multiple markets found in them, this study seeks to establish the current status of GM crops in Northern Nigeria and to accurately analyse the safety of the soon to be released transgenic cowpea resistant against *maruca* in Nigeria using *in silico* omics tool.

1.4 Aim of Study

The aim of this study is to establish a biosafety database of GM Crop under confined field trial and those from possibly available or illegally introduced GM crops in northern Nigeria.

The specific objectives are:

i. To survey the possible existence of GM Events in Soybean and Maize of Northern Nigeria using flow strip technique.

ii. Molecular characterization of possible transgene in commercialized maize, soybean and transgenic cowpea samples using Polymerase Chain Reaction (PCR).

iii. To assess the morphological characteristics between transgenic and non-transgenic cowpea.

iv. To carry out an *in silico* risk assessment of the transgenic cowpea based on allergenicity and toxicity test.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Genetically Modified Crops

According to *Siegfried (2012),* the first genetically modified crop is antibiotic-resistant tobacco plant and was produced in 1982 after a first field trials was carried out by engineering tobacco plants for herbicide resistance in 1986 at both France and the USA simultaneously (*Devos, et al., 2013*). In 1987, Plant Genetic Systems (Ghent, Belgium), founded by Marc Van Montagu and Jeff Schell, was the first company to genetically engineer insect-resistant (tobacco) plants by incorporating genes that produced insecticidal proteins from *Bacillus thuringiensis* (*Bt*). The Republic of China was the first country to allow commercialized transgenic plants, introducing a virus-resistant tobacco in 1992 which was withdrawn in 1997 (*Bagla, 2010*). FlavrSavr tomato was the first GM crop approved for sale in the U.S. in 1994. This GM tomato had a longer shelf life, because it took longer to soften after ripening (*Tabashnik, et al., 2008*).

2.2.1 Genetic engineering techniques

The techniques used for the engineering of crops gene are referred to as genetic engineering (GE) techniques. Modern plant breeding is a multi-disciplinary and coordinated process where a

large number of tools and elements of conventional breeding techniques, genetic engineering, bio informatics, molecular genetics and molecular biology are utilized and integrated. Basic GE tech niques are: microinjection, electroporation, gene guns, and agrobacterium (DANIDA, 2002).

2.3 GM crops and examples of modified traits

Several traits have been targeted for improvement by gene technology. The first targets included herbicide tolerance, disease and insect resistance, quality improvements (Dunwell, 1998) and tolerannce to abiotic stresses (drought). An example of herbicide tolerant GMOs is the Monsanto Roundup Ready® group of products that have been genetically modified by introducing a gene from the plant pathogen *Agrobacterium tumefaciens* strain CP4. These traits are providing economic benefits to the agrochemical industry, seed producers and farmers due to enhanced productivity (Imai, *et al.*, 2002). The production of vaccines in plants such as rice (Mercenier *et al.*, 2001), bananas or potatoes is currently under evaluation (Stoger, *et al.*, 2000). Some other GM crops and examples of modified traits are shown in appendix 1.

2.3.1 Genetically Modified Maize

GM maize is a maize crop whose genome has been altered. The first GM maize producing a *Bt* Cry protein was approved in 1996, this transgenic maize which killed European corn borer and related species; subsequent *Bt* genes were introduced that killed corn rootworm larvae (Hellmich and Hellmich, 2012). For Zea *mays*, 144 events have been found, some of which are highlighted in appendix 2.

2.3.2 Genetically Modified Soybean

A GM soybean is a soybean (*Glycine max*) that had a foreign DNA introduced into it using genetic engineering techniques. The first GM soybean was commercialized in the U.S. by Monsanto in 1994. In 2014, 90.7 million hectares of GM soy were planted worldwide (ISAAA, 2016). The genetic makeup of soybean gives it a wide variety of uses, thus keeping it in high demand. Some of the events of Soybean are highlighted in appendix 3.

2.4 Regulation of the Release of Genetically Modified Organisms

There is variation in the regulation of GM crops between countries especially between the USA and Europe (Law Library, 2017). One of the fundamental issues concerning regulation of GM crops is the labeling of GM products. Already, labeling of GM products is required in 64 countries (Hallenbeck, 2014). Whether a GM product is to be labelled or not, is largely dependent on the threshold GM content level (which varies between countries) or voluntary (Botha and Viljoen, 2009). For example, in South Africa, 31% of products labeled as GMO-free has a GM content less than 1.0% (Botha and Viljoen, 2009). In Europe, while all food containing greater than 0.9% of GM content must be labelled (John, 2010), it is voluntary in Canada and USA. In Brazil, GMOs are governed by a law that defines the concept of a GMO and sets rules for the laboratories that work with them (Law Library, 2017).

One key regulatory measures of GMOs is the adoption of the Cartagena Protocol in January 29, 2000, by the unanimous consent of 135 countries. The Cartagenal Protocol on Biosafety is a legally binding global protocol that seeks to contribute to ensuring the safe transfer, handling and

use of living modified organisms (LMOs) created through modern biotechnology (Sendashonga *et al*., 2005).

2.5 Biosafety of GM Crops

In order to determine whether a modified product is substantially equivalent, a test for unexpected changes is carried out by the manufacturer. This test normally includes toxicity and allergenicity tests. Results from such test is then assessed by the regulatory agency, such as the U.S. Food and Drug Administration. After which it is submitted to the regulators. Further analysis then focuses on the evaluation of the defined differences. Set of factors only peculiar to evaluating food and feed safety are used for assessing potential safety risks of the host plant, gene donor(s), and introduced protein(s). In a review by Kok and Kuiper (2003) seven main steps of a standard safety test were identified:

i. Study of the introduced DNA and the new proteins or metabolites it produces;
ii. Analysis of the chemical composition of the relevant plant parts, measuring nutrients, anti nu-trients as well as any natural toxins or known allergens;
iii. Assess the risk of gene transfer from the food to microorganisms in the human gut;
iv. Study the possibility that any new components in the food might be allergens;
v. Estimate how much of a normal diet the food will make up;
vi. Quantification of any nutritional or toxicological problems revealed by this data in light of data on equivalent foods;
vii. Additional animal toxicity tests if there is the possibility that the food might pose a risk.

2.6 Benefits of GM Crops

2.6.1 Health benefits

In addition to reduced use of pesticide, GM crops can increase the nutritional value of foods and enhance human health in various ways (Pocket, 2009):

i. Lower levels of infestation by insects reduces fungal and mycotoxin in maize.

ii. Rice nutritionally enhanced for betacarotene, would serve as a good source of vitamin A thereby saving millions of children from blindness every year.

Biotechnology processes can reduce presence of toxic compounds - e.g. cyanide in cassava.

2.6.2 Environmental Benefits of GM Crops

i. Cumulative reduction in pesticides usage – estimated 224,300 MT of active ingredients for the period 1996-2005. This has contributed to reduction of pesticide residue in foods and minimized impact on non-target organisms.

ii. Increased productivity per unit of land, minimizing encroachment into marginal lands, destruction of forests and pollution of fresh water resources (Pocket, 2009).

2.6.3 Pesticide use

There have been reduced pesticide inputs of 503 million kg pesticides by farmers planting GM crops since 1996 thereby leading to overall reduction in the environmental footprint of GM crops by 18.7%. Environmental footprint is a measure of the effect or impact a product, process, operation, an individual or corporation places on the environment, in this case, measuring the environmental effects of pesticides (Brookes and Barfoot, 2014).

2.6.4 Livestock farming

Feed grain usage as a percentage of total crop production ranges from 18% for wheat, 52% for sorghum, 70% for corn, 75% for oats, to more than 90% of oil seed meals (MacKenzie and McLean, 2002). There is high preference to corn grain and soybean meal as protein and energy source in both monogastric and ruminant diets with at least 65 million metric tons of GM corn grains used in livestock diets annually. While about 70 million metric tons of GM soybean meal are fed to livestock per annum (Gilbert, 2000).

2.7 Countries and their Experience: The Track Records of GM Crops

Several studies on GM crop adoption in various countries have been done. The multiple benefits derived from GM crops have been highlighted. Examples are the following:

2.7.1 India

Cotton is a very important crop for India, accounting for 30% of its agricultural GDP. However, due to the high incidence of pests, especially the cotton bollworms, India falls short of the world's average yield of cotton by 48%, an equivalent of 280 kg/ha (James, 2013). Indian farmers often lose up to 50-60% of their crop to the cotton bollworm (Hsiaoping, 2005). There have been acute reduction in cyclic infestation of bollworm due to the commercialization of *Bt* cotton in India since 2002 leading to the high ranking of India as the first in GM cotton production worldwide in 2013 (James, 2013).

2.7.2 United States

An estimated cost savings by farmers planting HT soybean was $71.3/ha in 2012, almost three times higher compared to the early years of adoption. The annual total national farm income

benefit from HT soybean has dramatically risen from $5 million in 1996, to nearly $6.07 billion in 2012 (Brookes and Barfoot, 2014). The US is estimated to have enhanced farm income from GM crops by $53.1 billion in the period 1996 to 2012 (Brookes and Barfoot, 2014).

2.7.3 Canada

HT canola has boosted the total canola production in Canada by 11% in 2012. Adopters of GM canola earned $446 million in 2012 (Brookes and Barfoot, 2014). The net increase in farm income by HT maize farmers in 2012 was $12.2 million (Brookes and Barfoot, 2014). Canada is estimated to have enhanced farm income from GM crops by $4.9 billion in the period 1996 to 2012 (Brookes and Barfoot, 2014).

2.7.4 Spain

Bt maize adoption in Spain in 2012 resulted in yield increases of 6.3% on average, the net impact on gross margin $320.3 per hectare (Yorobe, 2004). Farmers also experienced savings on pesticide use by $8.24/ha (Qaim and Khouser, 2013).

2.7.5 Australia

For 2012, Australian farmers planting IR cotton have significant cost savings of about $186-270/ha despite the high cost of technology. In 2012, net farm income at the national level was $766 million (Brookes and Barfoot, 2014).

2.7.6 Philippines

A common corn pest in the Philippines is the Asiatic corn borer which causes losses of up to 80% of production. Across the country, corn yield levels averaged only 2.8 tons per hectare. Philippine was the first country in Asia to commercialise *Bt* corn as food and feed crops. More than 10,000 hectares was covered for *Bt* crop plantings in the first year (2003) the crop was commercialised. Adoption of *Bt* corn in the Philippines provided the following benefits to small-scale farmers (James, 2013; Sankula, 2005):

i. Yield advantage of about 1.1 ton/ha or 30% yield increase over conventional corn hybrids.
ii. Pesticide cost reduction by as much as 56%.
iii. Profit gain of PhP10,132/ha (US$180), with PhP168/ha savings in insecticide costs.
iv. Increased net profitability by 4-7% during wet season, and 3-9% during dry season.
v. Premium price for *Bt* corn because of good quality grains.

Furthermore, socio-economic studies on GM corn also confirmed that it has positive impact on small and resource-poor farmers and corn producers in the Philippines.

2.7.7 China

The decreased in rice yield due to insect damage has been estimated to cost several billions of dollars worldwide. Rice is the most important crop in China, with the highest level of production accounting for 28% of the world's total production (Xue, *et al.*, 1996). In 2009, insect-resistant GM rice was approved for food, feed, and cultivation in China. Farm surveys conducted to establish whether farmers welfare improved by planting GM rice shows that small and poor farm households who adopted GM insect-resistant rice benefited by having higher crop yields and lower pesticide usage compared to non-GM adopters. GM rice yields were 6 to 9% higher

compared to conventional varieties and it required less pesticide input by as much as 80% or 16.77 kg/ha (Huang, *et al*., 2005).

2.7.8 South Africa

This African country has commercialised GM crops since 1998. To date, South Africa has comm ercialised *Bt* cotton (Bennett *et al*., 2006). The production records of *Bt* cotton for three growing seasons in South Africa showed that it yields were substantially higher (89–129%) than conventional cotton, especially under adverse climatic conditions (Stuart *et al*., 2015). In another three seasons of research involving small landholder adopters of GM crops in South Africa, Gouse (2013) found that GM herbicide tolerant (HT) maize adopters maize adopters spent 10-12 fewer days per season doing grueling weed pulling and hoeing than their conventional maize planting counterparts. Over the three years of the study, the average economic return was more than double that of conventional cotton, with returns of US$151/ha for *Bt* cotton and US$70/ha for conventional cotton. Health benefits were also found to be substantial with an estimated 30,000 fewer reported cases of pesticide poisoning (Stuart *et al*., 2015).

2.8 GMOs Detection Methods

The monitoring for the presence and amount of GM crops and products has spawned a call for analytical methods capable of detecting, identifying and quantifying either the DNA introduced or the protein(s) expressed in them (Gadani *et al*., 2000). There are three major steps in an all-inclusive GMO analysis technique: Detection, identification and quantification (Tripathi, 2005).

2.8.1 Detection

The objective of GMO screening is to determine if a product contains GMO or not. For this purpose, a detection method can be used. The result is a positive/negative statement (Kay and Van, 2001). Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results.

i. GMO detection by phenotypic characterization

Phenotypic characterization is possible if the targeted gene can be used to determine the absence or presence of a specific trait. This approach is referred to as bioassays. It can be done by germinating seeds in the presence of the herbicide of interest. Herbicide assays are considered to be accurate and inexpensive. Controls including seeds with or without the trait targeted are usually included in all samples (Tripathi, 2005). The accuracy relies on the germination: the higher germination the higher is the confidence level of the test.

ii. Molecular Detection of GMOs

Methods that target the inserted DNA and it expressed proteins have been developed and are widely used for detecting GMOs. This is because the target DNA can be purified and amplified by polymerase chain reaction (PCR). Protein-based methods rely on a specific binding between the protein and an antibody. The antibody recognizes the foreign molecule and binds to it; and in GMO detection assays the bound complex is successfully detected in a chromogenic (color) reaction. This technique is called ELISA (Enzyme Linked ImmunoSorbent Assays).

iii. Protein-based method of detection: Immunoassay

Immunoassay is the current method for detecting and quantifying a target protein associated with genetic modification. It can be used for qualitative and quantitative measurements over a range of concentrations. Different types of immunoassays including enzyme-linked immunosorbent

assay (ELISA), dipstick and lateral flow procedures are available for use in the field and in the laboratory.

2.8.2 Identification

The identification of any GM crop and product is very fundamental since they usually have exact appearance as anon-GM products. The purpose of GM event identification is to reveal how many different GMOs are present and if they are authorized or not. If there is a positive detection of GMOs, further analysis is required to discover which GMO it is and thus whether the GMO is approved within the EU. The only analytical methods, which unequivocally may enable identification of each GMO variety are methods based on PCR (Tripathi, 2005). Other possible methods of GM event identification include Enzyme Linked Immunosorbent Assay (ELISA) and Flow strips.

2.8.3 Quantification

If a food product has been shown to contain (one or more) authorized GMOs, then it becomes necessary to assess compliance with the set threshold level of regulation by the determination of the amount of each of the GMOs present in the individual ingredients from which the food item has been prepared (e.g. Maize flour). Quantification of any event expressed in Gm crops and products can be done by Conventional PCR and Real-time PCR techniques. While Conventional PCR measures the products of the PCR reaction at the end point in the reaction profile (Gadani *et al*., 2000), Real-time PCR is a system based on the continuous monitoring of PCR products.

2.9 Bioinformatics steps in assessing Allergenicity and Toxicity of a Protein

The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation and heat stability (DBT, 2008). Amino acid sequence homology comparisons need to be used to assess the extent to which a newly expressed protein is similar in structure to the known allergens to determine whether that protein has allergenic or cross-reactivity potential. Searches need to be conducted using various algorithms such as FASTA or $BLAST_P$ to predict overall similarities at sequence level (ICMR, 2008). While the FASTA and $BLAST_P$ programs compare amino acid sequences (i.e., primary protein structure) and provide optimum local sequence alignments, the results can be used to infer similarity of higher order structure. Proteins that share a high degree of similarity throughout the entire length are considered homologous and often these proteins share secondary structures and common three-dimensional folds (Pearson, 1996). The results of the search are used to identify recombinant proteins expressed in transgenic plants, which are to be tested by specific serum screens, and to identify the specific allergic populations that might be at risk, and should be used as serum donors for specific testing (Codex Alimentarius, 2003). The bioinformatics search is relatively straightforward and should markedly reduce the risk of transferring even a minimally cross-reactive protein. Hence, the choice of appropriate allergen database for search is vital for a reliable sequence comparison (Goodman *et al.*, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Sample materials

Grains of soybean and maize were randomly collected in brown labelled envelope from major markets across Northern Nigeria (Table 3.1). Grains were selected, planted and leaves from them were used to extract DNA according to Qiagen DNeasy Plant Mini Kit (2012) manufacturer's instruction. Before collection of Confined Field Trial (CFT) *Maruca* Resistant *Bt* Cowpea sample, a letter of introduction with identification number, NBMA/ODG/90 was obtained from the National Biosafety Management Authority (NBMA) and was issued to the Institute for Agricultural Research (IAR), Zaria. A total of 300g of seeds were obtained each for IT97KT, IT97KN and IT86-D1010 for analysis.

3.2 Methods

3.2.1 Study Area

The study area is Northern region of Nigeria with coordinate of Latitude: 10° 30' 59.99" N and Longitude: 7° 25' 59.99" E. The region is made up of nineteen states and zoned into three major geopolitical zones: North East, North West and North Central (UNICEF, 2018). As at 2007, the Northern Nigerian region was estimated at a population of 75,025,166 (Chidi, 2007). The 6 geopolitical zones of Nigeria is highlighted in Figure 3.1.

Table 3.1: States and the sampled markets

SN	States	Markets		
1	Kaduna	Kao Central market	Giwa market	Samaru Market
2	Kano	Dawanau Central market	Rimi market	Kofar Wabe market
3	Plateau	Mangu maize market	Farin Gada market	Bukuru urban market
4	Niger	Madala market	Bida market	Minna Market
5	Katsina	Bakori grains market	Dandume grain market	Kankara weekly market
6	Zamfara	Kaura Namoda market	Gusau central market	Talata Mafara market
7	Kwara	Ojaoba martket	Marab market	Offa market
8	Taraba	Mutum-biyu market	Abari market	Garba Shade market
9	Borno	Monday market	Kasua gamboru	Tashan baga
10	Adamawa	Mubi Grain markets	Jimeta Ultra-Modern market	Yola South Central market
11	Benue	Lessel	Gbajimgba	Makurdi central market
12	Abuja	Dutse's market	Bwari's	Gosa market
13	Gombe	Tudun Hatsi market	Gombe main market	Babbar kasuwa

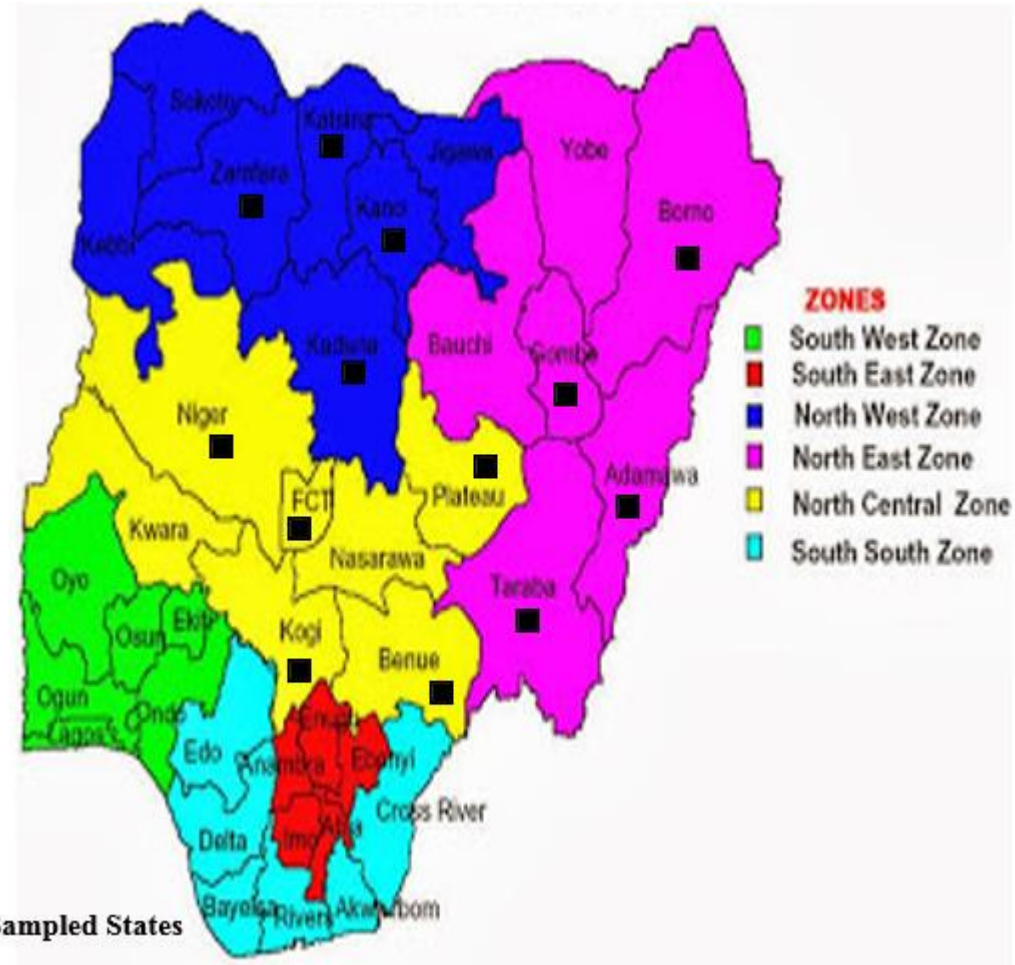
Sokoto
Katsina
Jigawa
Yobe
Zamfara
Kano
Borno
Kebbi
Kaduna
Bauchi
Gombe
Niger
Adamawa
FCT
Plateau
Kwara
Nasarawa
Taraba
Oyo
Osun
Ekiti
Kogi
Benue
Ogun
Ondo
Edo
Enugu
Ebonyi
Cross River
Delta
Imo
Abia
Bayelsa
Rivers
Akwa Ibom
ZONES
South West Zone
South East Zone
North West Zone
North East Zone
North Central Zone
South South Zone
Sampled States

Figure 3.1: The six geopolitical zones of Nigeria. Source: Study and Scholarships (2013).

3.2.2 Sampling Technique

A systematic random sampling technique was used in this study. A total of 2,430 grains were analysed. The sampling procedure is schematically presented below. Thirteen states including the FCT, Abuja, were purposively selected using a multi stage sampling procedure. These twelve states cover a wide variety of different types of grain. Three major markets were randomly selected in respective state.

3.2.3 Sample preparation and processing

Viable seeds of maize and soybean were washed thoroughly with distilled water to avoid contamination.

3.2.4 Flow strip analysis for the maize and soybean samples

Sampled maize and soybean were analysed using the immunochromatographic strip test method of TraitChek^{TM} (Romer Labs) based on the manufacturer's recommendation for the detection of

*Bt*1Ac (lot number 6M1053), *Cry1Ac* (lot number 6G1027), LibertyLink *PAT* (lot number 030023) and *Cry2A* (lot number 031157). Water was added to samples in a Mason- Type Jar. With the aid of transfer pipette, 0.5 ml of solution was transferred to a sample tube. A clean strip was placed into the sample tube. The observation was recorded after twenty minutes.

3.2.5 PCR analysis for the maize and soybean sample

The PCR technique is based on the enzymatic amplification of targeted sequences of DNA. 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 according to Ibrahim *et al.* (2017) with pre-incubation at 95 ^{0}C for 10 min, initial denaturation of 30 seconds at 95°C and annealing at 59 ^{0}C for 30 s. The cycles was repeated 50 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.

3.2.6 Experimental Set up

The setup of the experiment was done systematically. Each step in the experiment was sequentially followed (Figure 3.2).

3.2.7 Event characterization for the GM Cowpea

The protein produced in detected GM cowpea was characterized with respect to the three-dimensional X-ray crystal structure (Stallings *et al*., 1991). A range of analyses were carried out

to characterise the genetic modification in these transgenic plant which includes:

Bioinformatics analyses and plant morphology.

The forward and reverse primer sequence of *Cry1Ab* gene is presented in table 3.2.

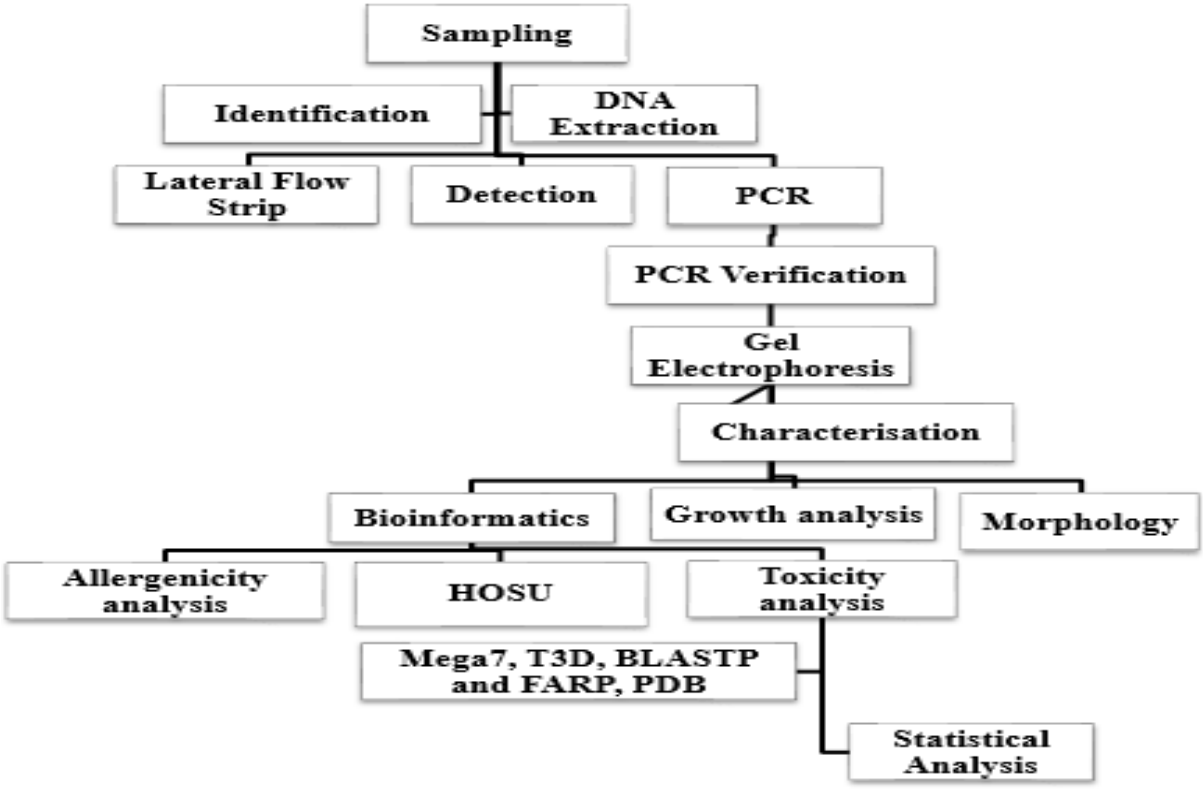

Figure 3.2: Scheme of the experimental procedure for the identification and analysis of transgenic event. *HOSU: History of Safe Use; PCR: Polymerase Chain Reaction; T3D: Toxins and Toxin Target Database;PDB: Protein Data Bank; BLASTP: Protein Basic Alignment Sequence Tool.*

Table 3.2: Selected Oligonucleotide Primers for PCR

Primer	Sequence (5'-3')	Target Gene	AS (bp)
Forward	5´-CAGGAACCAGGCCATCTCTA-3´	*Cry1Ab*	188
Reverse	5´-CGCTCCAAGCCAGTGTTGTA-3´	*Cry1Ab*	188

AS (bp): Amplicon Size (base pair)

3.2.8 Bioinformatics analysis and omics tools

The Omics tool updated version of 17 allergen and toxin data bases were used. These databases and their url is presented in table 3.3.

3.2.9 *In silico* analysis of the potential toxicity of Cry1ab gene expressed in *Maruca* resistant GM Cowpea

The potential toxicity assessment was run using Pan Pesticide Database (PPD) and *Toxin and Toxin Target Database* (T3DB). T3DB currently host a total of 42,471 toxins, 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 (Wishart, *et al.*, 2015) while PPD hosts 6,500 pesticides, insecticides and herbicides including toxicity, water pollution, ecological toxicity. Their url are presented in table 3.3. This assessment was focused on:

Whether the novel protein has a prior history of safe human consumption, eg Naturally-occurring *Bacillus thuringiensis* strains (Frederiksen *et al.,* 2006).

Sufficient similarity to proteins that have been safely use, eg transgenic plants containing Cry proteins (Betz *et al.,* 2000). Amino acid sequence similarity with known protein toxins and anti-nutrients; these were done in comparison of the Toxins and Toxin Target Database (T3D) and the PAN Pesticide Database (PPD). This database assembled from PRT_2009 consists of 7,176 sequences associated with known toxins.

The FASTA sequence alignment tool was employed, with the E-score set at $1x10^{-1}$ and according to the respective database cut-off score. The E-score (Baxevanis, 2005) represents the probability that a particular alignment is due to chance. Comparisons between highly homologous proteins should yield E-values approaching zero, indicating a very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity.

Table 3.3: Details of the 17 Allergen and Toxin Databases used for the *in silico* analysis of *Cry1Ab* gene

SN	DATABASE	URL
i	Food Allergy Research and Resource program	www.allergenonline.com
ii	National Centre for Biotechnology Information	www.ncbi.nlm.nih.gov
iii	Allergen Families (AllFam)	www.meduniwien.ac.at/allergens/allfam/
iv	Pan Pesticide Data base (PPD)	www.PesticideInfo.org
v	Interproscan Domain database	www.ebi.ac.uk/Tools/InterProScan/
vi	Allergome	http://www.allergome.org
vii	Structural Database of Allergenic Proteins	fermi.utmb.edu/SDAP
viii	Allermatch	http://www.allermatch.org
ix	Pubmed Data Base	http://www.ncbi.nlm.nih.gov/pubmed/
x	Allergen Database for Food Safety (ADFS)	http://allergen.nihs.go.jp/ADFS/

xi	Allerbase	http://196.1.114.46:1800/AllerBase/HTML/BLAST.html
xii	Universal Protein Resource (UniProt)	http://www.uniprot.org
xiii	IUIS Allergen Nomenclature (IUIS)	www.allergen.org
xiv	Food Allergens of Plant Origin (Protall)	www.ifr.bbsrc.ac.uk/Protall
xv	Toxins and Toxin Target Database (T3D)	http://www.t3db.ca/biodb/search/target_bonds/sequence
Xvi	WHO-IUIS Database	*http://www.allergen.org*
Xvii	Protein Structure Discovery (PSD)	http://www-bionet.sscc.ru/psd/

3.2.10 History of Safe Use (HOSU)

A review on the history of safe use (HOSU) of Cry1Ab protein was performed according to the principles described by Constable *et al.* (2007). This search was composed by reports for the *Bt* (source of the Cry1Ab protein) and three-domain (3_D) Cry proteins. This extrapolation was made since three-domain Cry proteins share high structural similarities among them, which are connected to their functions and biological activities (Sanahuja *et al.*, 2011; Hammond and Koch, 2012). Thus, the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) was 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*”.

3.2.11 Bioinformatics analysis of the potential allergenicity of the transgenic Cowpea

Fifteen database systems including AllergenOnline version 17.0 database (updated in February 18, 2017) were employed for this study. FASTA (or $BLAST_P$) search with a threshold concern of 35% identity as a primary bioinformatics method as highly recommended was used (Gurinder *et al.,* 2011). Based on the information of scientific literature for sequence identities of clinically demonstrated cross-reactivities, it appears that 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 (Goodman *et al*., 2005), to compare against a well-founded allergen database. The sequence alignment search for this study were carried out using scoring matrix of BLOSUM62 with FASTA3 and $BLAST_P$ 2.2.27 algorithm of the data base presented in Table 3.3.

3.2.12 Three-Domains (3-D) *in silico* allergenicity assessment

The cry proteins were evaluated for presence of domains similar to those of allergen proteins and toxins for the possibility of allergenic cross reactivity even in the absence of sequence homology. Interproscan (<http://www.ebi.ac.uk/Tools/ Interproscan/>) was used to deduce the domains present in the Cry proteins. E score is a statistical measurement of the probability that the alignment was only due to chance, rather than an alignment due to ‘real’. The domains from the two sources were compared for potential similarity at the domain level.

3.2.13 Amino acid sequence homology search

The bioinformatics search for allergen sequence matches was conducted according to Goodman *et al*. (2005). Search was done for 80 amino acid alignment, 8 amino acid alignment and 6 amino acid alignment, looking for identities greater than 35% using the current version of 17 databases (Table 3.3). A sequence similarity search was conducted with one amino acid sequence of the Cry1Ab protein using $BLAST_P$ 2.2.20 with an e score cutoff of 1.0 (Delaney *et al*., 2008). A total of 8,996,415 sequences were searched. Sequence annotations of proteins with similarity to the Cry1Ab protein sequence were inspected manually to identify known allergens or toxic proteins.

3.2.14 Sliding 80mer/ windows search

This was done using the AllergenOnline database. To be consistent with Codex 2005, the calculation of the cutoff value for a match was changed to > 35%. The E-score cutoff for the sliding 80mer search was changed from 100 to 10 as explained on AllergenOnline website page.

3.2.15 Sliding 8mer/ 6mer windows search

The sliding 8mer/6mer windows search was carried out using the Allermatch database. Allermatch compares the amino acid sequence of a query protein with sequences of allergenic proteins. This allergen database predicts the potential allergenicity of a protein using

bioinformatics approaches, as recommended by the Codex Alimentarius and FAO/WHO expert consultation on the allergenicity of Genetically Modified Products (Fiers, 2004).

3.2.16 Algorithm

Algorithm used for comparisons was $BLAST_P$ 2.2.27+ and the scoring matrix was BLOSUM62. All comparisons were run from August to October, 2017. Sequence search was carried out to specifically assess the allergenic potential of cry proteins. *Cry1Ab* sequences were subjected to full sequence alignment using E-value cutoff of 1.0 for detection of identity >70% for the complete sequence similarity and >35% in a window of 80, 8 and 6 amino acids. In the NCBI database, comparisons were run using keyword limits, and observing carefully specific details of alignment (E-scores 35%). For each sequence tested, the keywords "allergen", "toxin" or "antinutrient" were used independently. The criteria particularly determined for this work were based on the study of Moran *et al.* (2014).

3.2.17 Decision tree

To advance the strategy for allergy related safety assessment of genetically transformed foods, a revised decision tree approach set forth by the Food and Agricultural Organization/World Health Organization (FAO/WHO, 2001).

3.2.18 Judgment of results

i. Full FASTA sequence alignment/ search

Alignments with high identity scores may indicate a potential 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 (Aalberse, 2000). The extent of similarity was evaluated by the percent identity and expectation score (E-score). E-score much smaller than 1 (e.g 1×10^{-25}) indicate a highly significant alignment, probable evolutionary relationship and most importantly, a high degree of structural similarity. E-score higher than one indicates a low degree of similarity between the query sequence and the sequence from the database. The calculated e-score depends on the overall length of joined (gapped) local sequence alignments, the quality (percent identity, similarity) of the overlap and the size of the database (Hileman *et al.*, 2002).

ii. Sliding 80mer windows identity search for allergenicity and toxicity

More than 35% identity in the amino acid sequence of the expressed protein (i.e. without the leader sequence, if any), using a window of 80 amino acids and a suitable gap penalty (using Clustal-type alignment programs or equivalent alignment programs) was recommended and used.

iii. Sliding 8/6mer windows identity search for allergenicity and toxicity

If any of the identity scores equals or exceeds 35%, this was considered to have significant homology within the context of this assessment approach (Gendel, 1998a; Gendel, 1998b).

3.3 Morphological characterization: Line IT86D1010, IT97KT and IT97KN

The plant morphology was carefully characterised using the following parameters: Seed appearance (eye spot, shape, size and texture) and Leaf appearance (shape, size and texture).

3.4 Statistical Analysis

Values were expressed as mean ± SD. Data analysis was carried out using Statistical package for Social Sciences (SPSS) version 21. Data was subjected to one way Anova. Values with $p < 0.05$ was considered statistically significant. Duncan Multiple Range Test (DMRT) was further used for comparison of the means.

CHAPTER FOUR

4.0 RESULTS

4.1 Survey on the Status of maize and soybean crops in Northern Nigeria

4.1.1 Detection of Possible Transgenic Maize and Soybean using the Flow Strip Detection Technique

The results for the flow strips of traits: *RUR HS, Liberty Link PAT, Cry2A/1Ac* and *Bt1Ac* to detect possible events in maize and soybean samples were negative (Plate I and II), indicating absence of any possible event. The test line on the strips used to test the soybeans and maize

samples from major markets across the states were not visible while only the control line were visible which shows that the traits tested for were not in the samples collected from the commercial markets of these Northern states (Plate I a and b).

The PCR analysis of the samples obtained from one markets in the Northern region of Nigeria following the flow strip detection technique using the appropraite *bt* strips also confirmed the absence of the foreign gene, *Cry1Ab, Pat,* and *Cry2A/1Ac* Plate II (a, b and c). The gel photo of the PCR products generated using agarose gel electrophoresis gave no visible band in all the sample lanes. PCR analysis using specific primers of *Cry1Ab, pat* and *Cry2A/1Ac genes* all gave no band. Similar analysis carried out for other genes also gave the same result.

A total of 12 Northern Nigeria states were surveyed from between March, 2016 to June, 2017. The result of the survey is presented in table 4.1 (a, b and c). The summary of the flow strip test and PCR detection results shows a negative result for all sampled locations.

Plate I a: Flow strips for *RUR HS, LibertyLink PAT, Cry2A/1Ac* and *Bt1Ac* traits showing negative results for soybean samples.

Plate I b: Flow strips for *RUR HS, Liberty Link PAT, Cry2A/1Ac* and *Bt1Ac*traits showing negative results for maize samples

Plate II a: Gel photo of PCR products amplified using specific primers of *Cry1Ab*. Lane M: 1Kb DNA ladder; Lane 1 -3: Suspected positive samples Lane 4: Control sample.

Plate II b: Gel photo of PCR products amplified using specific primers of *Pat.* Lane M: 1Kb DNA ladder; Lane 1 -3: Suspected positive samples Lane 4: Control sample.

Plate II c: Gel photo of PCR products amplified using specific primers of *Cry2A/1Ac.* Lane M: 1Kb DNA ladder; Lane 1 -3: Suspected positive samples Lane 4: Control sample.

Table 4.1a: Event identification for maize and soybean samples in North Central Zone

Location	Sample Label	Flow strip and PCR detection results				Inference
		PAT	***Bt1Ac***	**Cry1a*c***	***Cry2a***	
Abuja	au1	-	-	-	-	Absent
	au2	-	-	-	-	Absent
	au3	-	-	-	-	Absent
	au4	-	-	-	-	Absent
	au5	-	-	-	-	Absent
	au6	-	-	-	-	Absent
	au7	-	-	-	-	Absent
	au8	-	-	-	-	Absent
	au9	-	-	-	-	Absent
Benue State	bn1	-	-	-	-	Absent
	bn2	-	-	-	-	Absent
	bn3	-	-	-	-	Absent
	bn4	-	-	-	-	Absent

	bn5	-	-	-	-	Absent
	bn6	-	-	-	-	Absent
	bn7	-	-	-	-	Absent
	bn8	-	-	-	-	Absent
	bn9	-	-	-	-	Absent
Kogi State	kg1	-	-	-	-	Absent
	kg2	-	-	-	-	Absent
	kg3	-	-	-	-	Absent
	kg4	-	-	-	-	Absent
	kg5	-	-	-	-	Absent
	kg6	-	-	-	-	Absent
	kg7	-	-	-	-	Absent
	kg8	-	-	-	-	Absent
	kg9	-	-	-	-	Absent
Niger State	ng1	-	-	-	-	Absent
	ng2	-	-	-	-	Absent
	ng3	-	-	-	-	Absent
	ng4	-	-	-	-	Absent
	ng5	-	-	-	-	Absent
	ng6	-	-	-	-	Absent
	ng7	-	-	-	-	Absent
	ng8	-	-	-	-	Absent
	ng9	-	-	-	-	Absent

(+) = positive; (-) = negative; au = bn = kg = ng = first and third letters of respective sampled state.

Table 4.1b: Event identification for maize and soybean samples in North East Zone

Location	**Sample Label**	**Flow strip and PCR detection results**				**Inference**
		PAT	***Bt1Ac***	**Cry1a*c***	***Cry2a***	
Adamawa State	aa1	-	-	-	-	Absent
	aa2	-	-	-	-	Absent
	aa3	-	-	-	-	Absent
	aa4	-	-	-	-	Absent
	aa5	-	-	-	-	Absent
	aa6	-	-	-	-	Absent
	aa7	-	-	-	-	Absent
	aa8	-	-	-	-	Absent
	aa9	-	-	-	-	Absent
Borno State	br1	-	-	-	-	Absent
	br2	-	-	-	-	Absent
	br3	-	-	-	-	Absent

	br4	-	-	-	-	Absent
	br5	-	-	-	-	Absent
	br6	-	-	-	-	Absent
	br7	-	-	-	-	Absent
	br8	-	-	-	-	Absent
	br9	-	-	-	-	Absent
Gombe State	gm1	-	-	-	-	Absent
	gm2	-	-	-	-	Absent
	gm3	-	-	-	-	Absent
	gm4	-	-	-	-	Absent
	gm5	-	-	-	-	Absent
	gm6	-	-	-	-	Absent
	gm7	-	-	-	-	Absent
	gm8	-	-	-	-	Absent
	gm9	-	-	-	-	Absent
Taraba State	tr1	-	-	-	-	Absent
	tr2	-	-	-	-	Absent
	tr3	-	-	-	-	Absent
	tr4	-	-	-	-	Absent
	tr5	-	-	-	-	Absent
	tr6	-	-	-	-	Absent
	tr7	-	-	-	-	Absent
	tr8	-	-	-	-	Absent
	tr9	-	-	-	-	Absent

(+) = positive; (-) = negative; aa = br = gm = tr = first and third letters of respective sampled state.

Table 4.1c: Event identification for maize and soybean samples in North West Zone

Location	**Sample Label**	**Flow strip and PCR detection results**				**Inference**
		PAT	***Bt1Ac***	**Cry1a*c***	***Cry2a***	
Kaduna State	kd1	-	-	-	-	Absent
	kd2	-	-	-	-	Absent
	kd3	-	-	-	-	Absent
	kd4	-	-	-	-	Absent
	kd5	-	-	-	-	Absent
	kd6	-	-	-	-	Absent
	kd7	-	-	-	-	Absent
	kd8	-	-	-	-	Absent
	kd9	-	-	-	-	Absent
Katsina State	kt1	-	-	-	-	Absent
	kt2	-	-	-	-	Absent

	kt3	-	-	-	-	Absent
	kt4	-	-	-	-	Absent
	kt5	-	-	-	-	Absent
	kt6	-	-	-	-	Absent
	kt7	-	-	-	-	Absent
	kt8	-	-	-	-	Absent
	kt9	-	-	-	-	Absent
Kano State	kn1	-	-	-	-	Absent
	kn2	-	-	-	-	Absent
	kn3	-	-	-	-	Absent
	kn4	-	-	-	-	Absent
	kn5	-	-	-	-	Absent
	kn6	-	-	-	-	Absent
	kn7	-	-	-	-	Absent
	kn8	-	-	-	-	Absent
	kn9	-	-	-	-	Absent
Zamfara State	zm1	-	-	-	-	Absent
	zm2	-	-	-	-	Absent
	zm3	-	-	-	-	Absent
	zm4	-	-	-	-	Absent
	zm5	-	-	-	-	Absent
	zm6	-	-	-	-	Absent
	zm7	-	-	-	-	Absent
	zm8	-	-	-	-	Absent
	zm9	-	-	-	-	Absent

(+) = positive; (-) = negative; kd = kn = kt = zm= first and third letters of respective sampled state.

4.2 Identification of the event present in the transgenic Cowpea

4.2.1 The flow strip event detection

The flow strip analysis result revealed a positive test line for the presence of Cry1Ab gene in the transgenic cowpea. No positive test line was observed for the other genes (Plate III).

4.2.2 PCR Event identification

The Gel photo of the PCR products gave a visible band in the IT97KT lane. No visible band was observed in both the lane of IT86D1010 and IT97KN (Plate IV).

4.3 *In silico* Safety Assessment of the Protein Expressed in the Transgenic Cowpea using NCBI BLAST$_P$ Omics Tool

The BLASTP search results revealed that the protein coded by *Cry1Ab* gene showed no homology with any allergens and toxins (Appendix 4) in the following NCBI Entrez:

Non-redundant protein sequences nr

Refrence protein (ref_seq) database

UniprotKB/Swiss-prot database

Protein data bank (pdb) database

Transcriptome Shotgun Assembly protein (tsa_nr) data base.

4.4 *In silico* Toxicity Assessment of *Cry1Ab* gene

4.4.1 History of Safe Use (HOSU)

More than 1,800 papers (from 1959 to 2017) were reviewed for this study. This search revealed that the protein has a long history of safety (Table 4.2).

Plate III: Flow strips for *Cry1Ab* gene showing positive results for *maruca* resistant transgenic cowpea and negative for the non-transgenic cowpea line.

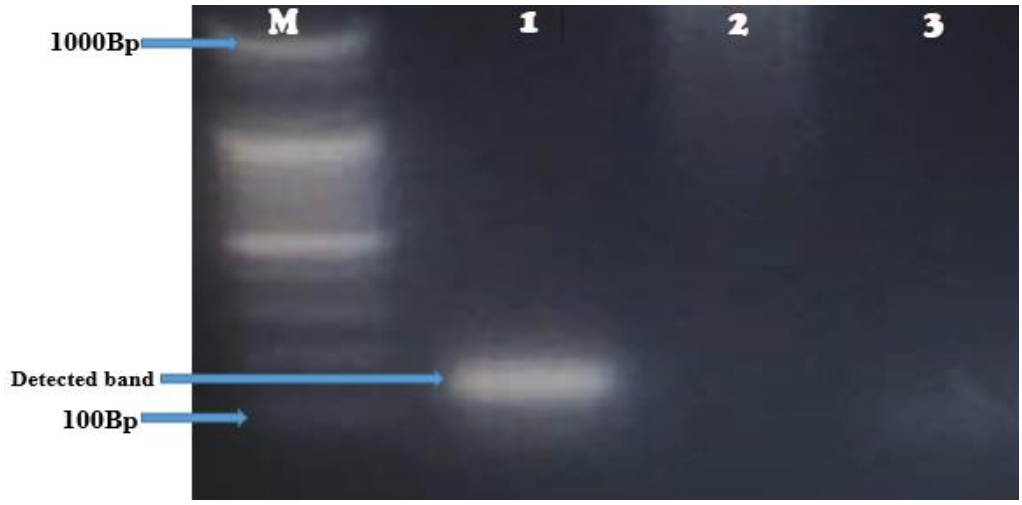

Plate IV: Gel photo of PCR products of *cry1Ab* gene from cowpea sample. Lane M: 1000 Bp marker; Lane 2: transgenic cowpea; Lane 3: ddH2O; Lane 4: conventional cowpea.

Table 4.2: History of Safe Use (HOSU) of Cry proteins using the PubMed and Protal Database

Cry Protein	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 honey bee	Niu *et al.*, 2017
Cry1Ac/2Ab	CD	Negligible exposure of phloem sucking hemipterans	Meissle, 2017
Cry1Ab/2Aj	r_m	No lethal or sub lethal effects	Zhang *et al.*,2017

NS	NSD	No adverse effect on the MCC	Liu *et al.*, 2017
NS	NSD	No significant effects on soil health	Zhaolei *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
NS	Safe	No significant harmful impact has been reported.	Yaqoob *et al.*, 2016
NS	NSD	Cry proteins are unlikely to function as adjuvants	Joshi *et al.*, 2016
Cry1Ac	NSD	No adverse impacts of *Bt* on species abundance, diversity and community dynamics.	Navasero *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
Cry1Ac	RB	No detected Cry protein in the soil 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	Has no significant long-term (90 day) toxic effects	Wang *et al.*, 2013

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.

Continuation of Table 4.2

Cry Protein	Result	Findings	Reference
1Ac/Aa/1Ca	NSD	*Bt* rice have no detrimental effects on any of the physiological processes of BPH.	Mannakkara *et al.*, 2013
Cry1Ac-M	NSD	*BT*-38 maize is as safe as it conventional maize.	Liu *et al.*, 2012
Cry3Bb1	NSD	No adverse effects on various NTO	Devos *et al.*, 2012

Cry1C	NSD	Cry1C protein is not a potential allergen or toxin	Cao *et al.,* 2010
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 at high concentrations	Bondzio *et al.,* 2008
NS	NSD	No uniform effects of *Bt* crops on the functional guilds.	Wolfenbarger *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
Cry1F	NSD	*Cry1F* maize was equivalent to non-GM maize	Herman *et al.,* 2004
Cry3Bb1	NSD	MON 863 is safe and nutritious as it non GM corn	George *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
Cry1Ab	AS	No toxicity was observed	Noteborn *et al.*1994
1Aa/1Ab	AS	No evidence of toxicity/ infectivity/ pathogenicity	David, 1989
Cry1Aa/b/c/2A	NSD	Not a virulent or invasive mammalian pathogen.	Hadley *et al.,* 1987
NS	NSD	Crystal delta endotoxin, produced no pathological effects by oral administration.	Thomas and Ellar, 1983
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.

4.4.2 Pan Pesticide Database (PPD) full sequence search

The seven factors based toxicity search gave negative result for the cry protein (Table 4.3, Appendix 5). None of the proteins met the criteria for suspecting the toxic cross-reactivity. Domain-domain search revealed no similarities at the domain level of any form of known toxins.

4.4.3 Toxin and Toxin Target Database (T3D) potential toxicity assessment

The results of the protein search after the cry1Ab protein were subjected to the analytical omics tool of T3D showed no cross reactivity, similarity or identity with any of the known toxins (Appendix 6). The closest E-score obtained was 6.42.

4.5 *In silico* Allergenicity Assessment of the Cry1Ab Protein

4.5.1 Transgenic cowpea event full sequence alignment using the omics tool of 14 databases

The $BLAST_P$ 2.2.29+ algorithm of all the database omics tool used revealed that none of the proteins met the criteria for suspecting the allergenic cross-reactivity (Table 4.4, Appendix 7). The Maximum Identity Score was below the level that is likely to indicate cross-reactivity (i.e., <35%) as recommended by the Codex Alimentarius guide line of WHO. The E-score for the query protein, Cry1Ab was far higher than the cut-off, 1.0 (Table 4.4).

The highest MIS found with any of the protein in the allergen database was with major pollen allergen (Accession Number: ADK39021.1) at 27.6% MIS. This MIS value is no way close to the percentage cut off as recommended by WHO. The results of this study showed no similarity between the Cry1Ab protein and allergen proteins in full sequence matching also. The full FASTA alignment between the query sequence (Cry1ab protein) and all the allergens in the SDAP also revealed a MIS much lower than 35% (Table 4.4). The results of this study confirmed no similarity between the Cry1Ab protein and allergen proteins in full sequence matching.

Table 4.3: PPD Toxicity information for *Bacillus thuringiensis* Cry1Ab protein

Factors	Result	Remark
PAN Bad Actor Chemical	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

Table 4.4: Full Sequence Homology Global Alignment of Cry1Ab protein based on FAO/WHO Allergenicity rules using the omics tool of selected databases with FASTA 35.0/3.45 Algorithm and Blossom 62 scoring matrix.

Database	Allergen	GBank/ Uniprot	MI (%)	E score obtained	E score NIA cut off		BS	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

FARRP[c]	*Pollen allergen*	ADK39021.1	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
SDAP[c]	Pen ch 200101	AAB34785	-	2.01	-	20/995	-	CNM
SDAP[d]	Bra n 1	P80208	-	2.61	-	26/995	-	CNM
SDAP[e]	Asp f 5	CAA83015	-	5.83	-	58/995	-	CNM
SDAP[f]	Car b 1032	CAB02217	-	2.71	-	27/995	-	CNM
SDAP[g]	Cyn d 23	AAP80170	-	1.71	-	17/995	-	CNM

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

4.5.2 Allfam database

Sequence search using the AllFam database also confirmed Cry1Ab protein to be non-allergenic.

No sequence matches was found, No hit was also found.

4.5.3 Sliding 80mer windows search for potential allergenicity of Cry1Ab protein

An alignment of 1076 80mers was done. No hit was found (Table 4.5, Appendix 8) when the sliding 80mer search was conducted using cut off MIS of > 35% (with E-score changed from 100 to 10) as recommended by Codex (2003). There were no matches greater than 35% sequence identity (Table 4.5, Appendix 8). 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 (CAN: P15252) while the lowest identical amino acid in every 80 amino acid count was 8 with MIS of 10% and was found at 80 amino acid range of 1-80 which corresponded to Plaor1.0101 (ACN: ABY21305).

4.5.4 8mer exact match

Further search using 8mer exact match confirms that Cry protein is not a member of the allergen family (Table 4.6).

4.5.5 Six Amino acid exact word-match of Cry1Ab with known allergen sequence

The highest 6 amino acid exact word-match obtained is 1 (Table 4.7, Appendix 9), giving a percentage identity of 0.09% as against the 35% cut-off set by WHO. The protein did not meet any of the criteria to be tagged an allergen. The six amino acid exact wordmatch conducted using the Structural database also confirm cry1ab to be toxic free (Table 4.7).

Table 4.5: Sequence identity search of Cry1Ab protein in SDAP FASTA alignments for an 80 amino acids sliding window using FASTA 3.45 Algorithm

Database	Hit Found	GB/uniprot	Match Range	IA 80 Count	MI (%)	MI Cut-Off	Inference
AllergenOnline	NHF	NHF	NHF	NHF	NHF	>35%	CNM
Allermatch	NHF	NHF	NHF	NHF	NHF	>35%	CNM
SDAP[a]	Plaor1.0101	ABY21305	1-80	8	10	>35%	CNM

SDAP[b]	Eurm3	O97370	5 - 84	10	12.5	>35%	CNM
SDAP[c]	Ligv1	O82015	61 - 140	20	25.0	>35%	CNM
SDAP[d]	Alta2	AAD00097	91 - 170	17	21.25	>35%	CNM
SDAP[e]	Bosd8	AAA30478	131 - 210	16	20.0	>35%	CNM
SDAP[f]	Fage1	O9XFM4	150 - 229	12	15	>35%	CNM
SDAP[g]	Gald4	P00698	201 - 280	13	16.25	>35%	CNM
SDAP[h]	Pench20.010	AAB34785	282 - 361	16	20	>35%	CNM
SDAP[i]	Musa2.0101	O8VXF1	317 - 396	21	26.25	>35%	CNM
SDAP[j]	Bran1	P80208	343 - 422	18	22.5	>35%	CNM
SDAP[k]	Hevb1	P15252	415 - 494	22	27.5	>35%	CNM
SDAP[l]	Derp3	P39675	921 - 1000	13	16.25	>35%	CNM

IA80 count: Identical Amino Acid in every 80 Count; AA: Amino acid; CNM: Criteria Not Met NMF: No Matches Found; NHF: No Hit Found; NAA: Not an Allergen

Table 4.6: 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

Table 4.7: Six amino acid exact word match search

Database	Allergen	6-AA EWM	MI (%)	% Cut off	Inference
WHO-IUIS[a]	wi_Tri_a_34	1	0.09	35%	NA
WHO-IUIS[b]	wi_Sal_k_1_d	1	0.09	35%	NA
WHO-IUIS[c]	i_Jun_v_1_b	1	0.09	35%	NA

WHO-IUIS[d]	wi_Eur_m_14	1	0.09	35%	NA
UniProt/ WI[a]	al_Phl_p_11	1	0.09	35%	NA
UniProt/ WI[b]	al_Lol_p_11	1	0.09	35%	NA
UniProt/ WI[c]	al_Jun_v_1_a	1	0.09	35%	NA
UniProt/ WI[d]	al_Jun_a_1_b	1	0.09	35%	NA
SDAP[a]	Blot1.0201	1	0.9	35%	CNM
SDAP[b]	PhAA1	1	0.9	35%	CNM
SDAP[c]	CupA1	1	0.9	35%	CNM
SDAP[d]	Junv1.0102	1	0.9	35%	CNM

[a,b,c…:] different output; NA: Not Allergenic; ID: Identity; %EWM: percentage exactword match; WI: WHO-IUIS; CNM: Criteria for Allergenicity Not Met; MI: Maximum Identity; CNM: Criteria for Allergenicity Not Met; N: Number; AA: Amino Acid

4.6 Cry protein Three Domain (C3_D) Toxicity and Allergenicity Assessment

The C3_D *in silico* analysis using the Interproscan omics tool did not reveal any match to any of the allergen domain. The galactose binding domain-like (Figure 4.1 a - c) was not identified with any toxins or allergens repeat (Figure 4.1 a - c).

4.7 Morphological Characterization of the three Cowpea lines: Line IT97KT, IT86D1010 (Transgenic), and Line IT97KN (Non-Transgenic)

There was no morphological differences between the transgenic cowpeas (IT86D1010 and IT 97KT) and the non-transgenic cowpea (IT97KN). Seeds developed a kidney shape (Plate V). The seed colour is white while the eyespot is black for all the three lines. There was an alternate trifoliolate leaf development in all the three lines. The open display of flowers above the foliage and the presence of floral nectaries were peculiar to the three lines at 40 DAP (Plate VI).

4.7.1 Height analysis of the three cowpea lines

No significant difference ($p \geq 0.05$) exist in the plant height at 10 DAP although significant ($p < 0.05$) difference exist in the plant height at 20, 30 and 40 DAP. Line IT86D1010 had the highest crop height throughout the growth season (Table 4.8a and 4.8b) followed by line IT97KT while line IT97KN had the lowest crop height throughout the growing season.

4.7.2 Weight analysis of the three cowpea lines

The Plant weight was significantly ($p \leq 0.005$) different between the three crop lines at 10 DAP. Line IT86D1010 had the highest weight (5.10g) followed by line IT97KT (2.97g), while line IT97KN had the lowest weight (2.27g) at 10 DAP. No significant ($p \leq 0.005$) difference exists at 20, 30 and 40 DAP.

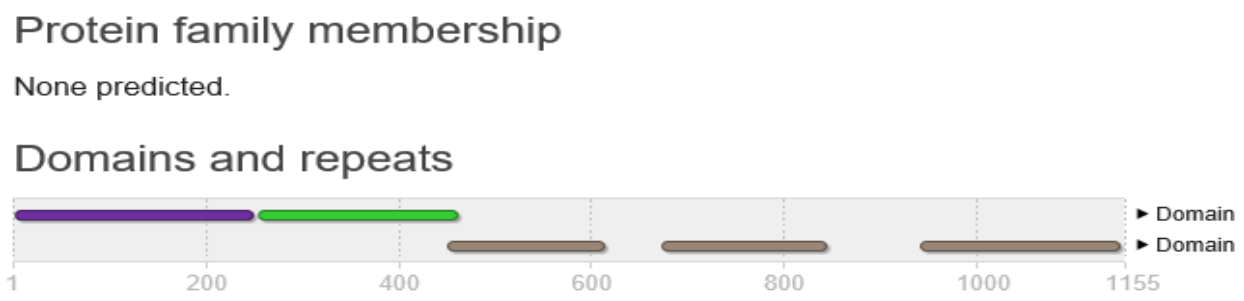

Figure 4.1 a: Domains and repeats

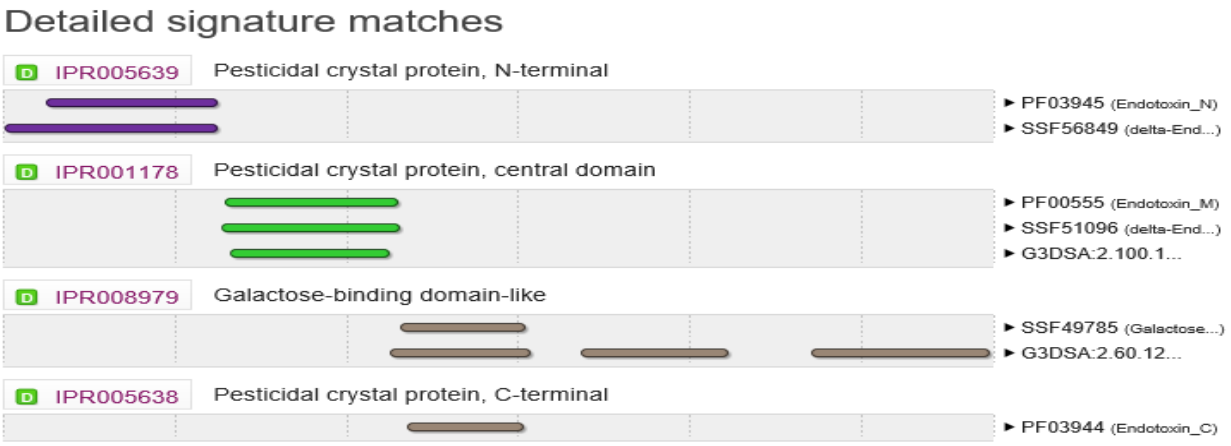

Figure 4.1 b: Detailed signature matches

Figure 4.1 c: Interproscan output result search: No matches was found between the cry protein and any of the speculated domain

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

During the growth periods, the seed coat was observed to be smooth and green for all the three lines. At germination, emergence was epigeal for all the three lines (as it is in common bean and lupin). All the lines were erect and growth habit was observed to be fairly determinate. There was an alternate trifoliolate leaf development in all the respective three lines. The open display of

flowers above the foliage and the presence of floral nectaries were peculiar to the three lines. Plate VI shows the picture of the three lines at 40 days after planting (DAP).

4.7.1 Height analysis of the three cowpea lines

No significant difference ($p \geq 0.05$) exist in the plant height at 10 DAP although significant ($p < 0.05$) difference exist in the plant height at 20, 30 and 40 DAP. Line IT86D1010 had the highest crop height throughout the growth season (Table 4.8a and 4.8b) followed by line IT97KT while line IT97KN had the lowest crop height throughout the growing season.

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ALP: Affected Leaf Plant; DAP: Days AfterPlanting

Plate VI: Leaves of three different lines of Cowpea. Line IT86D1010 and IT97KT are transgenic while line IT97KN is non-transgenic

Table 4.8a: Crop Growth Rate by Height

Crop lines	Crop Growth Rate by Height (gG^2 $dayG^1$)			
	10 DAP	**20 DAP**	**30 DAP**	**40 DAP**
IT86D1010	161.5000±19.092[a]	266.5000±9.192[a]	286.0000±4.243[a]	285.5000±4.950[a]
IT97KT	142.0000±16.971[a]	229.0000±4.243[b]	250.5000±4.950[b]	250.0000±5.657[b]
IT97KN	132.0000±4.243[a]	213.5000±10.607[b]	219.0000±12.728[c]	219.0000±11.314[c]

N=2; [abc]Values within the treatment group in the same column followed by same superscript (s) are not significantly different at (p≤0.05) according to DMRT;DAP: Days After Planting; ±: SD; CGRW: Crop Growth Rate by Height*

Table 4.8b: Growth Rate by Weight

Crop lines	Crop Growth Rate by Weight (gG^2 $dayG^1$)			
	10 DAP	**20 DAP**	**30 DAP**	**40 DAP**
IT86D1010	5.10±0.10[a]	11.60±0.20[a]	9.73±2.81[a]	12.93±4.21[a]
IT97KT	2.97±0.06[b]	8.80±0.10[a]	9.67±0.40[a]	13.77±0.31[a]

IT97KN	2.27±0.06[c]	26.40±34.73[a]	7.23±0.59[a]	9.20±0.30[a]

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

CHAPTER FIVE

5.0 DISCUSSION

Before now, the presence or absence of GM crops commercially in our markets have not been scientifically ascertained. Fear about the presence or absence of GM Crop in the Northern region

of Nigeria can only be justified by scientific research since transgenic and non-transgenic crops have the same appearance. Northern Nigeria has been proven over the years to serve as the Grain house of the country. Thus, detecting transgenic grains in this region may indirectly cover for other regions of the country. Though the detection of transgenic event in the commercial samples gotten from selected markets of Northern Nigeria indicated negative, similar studies carried out in Serbia by Gordana *et al.* (2014) and Orlandi *et al.* (2002) on detection of GM crops in animal feeds showed the presence of the transgene in contrast to another study carried out in Iran by Maryam *et al.* (2013). Though border States like Katsina and Adamawa State gave negative results as in others, Soybean obtained from Samaru market, Kaduna State initially showed confusing result. When further analysis by PCR was carried out on the sample, it was shown clearly that the samples were free of any foreign protein.

In this study, a scientifically based two tiered, weight-of-evidence strategy was adopted according the International Food Biotechnology Committee (ILSI) (Delaney *et al.,* 2008) to assess the safety of the event. Similar studies carried out in Iran by Najaf *et al.* (2015) on allergenicity assessment of Novel GM Foods confirmed *Cry1Ab* protein safe and nontoxic. Analysis by HOSU indicates that *Cry1Ab* undergoes rapid degradation. The rapid and extensive *in vitro* degradation of Cry1, Cry2, and Cry3 classes of proteins has been reported by Betz *et al.* (2000). The demonstrated rapid degradation of Cry1, Cry2, and Cry3 proteins consequently means that there is minimal likelihood of the protein inducing allergic reactions upon consumption since the potential for absorption is greatly reduced. Findings by Astwood *et al.* (1996) that food allergens often stays longer in gastrointestinal model, whereas non allergenic food protein degrades rapidly in simulated gastric fluid suggest that the Cry1Ab event is safe.

The variation in plant height among the three cowpea lines was in conformity with the previous findings of Nkaa *et al.,* (2014). Further reasons could also be attributed to the variation in the morphological status of the plant leaf. For instance, the plant leaf of the transgenic cowpea was seen to be very green and robust at 40 DAP while that of the conventional cowpeas was not (Plate VI). Since the rate of photosynthesis is highly dependent on surface area, it is possible that there was higher rate of photosynthesis in the GM cowpea than the non-GM cowpea thereby resulting in differences in the rate of photosynthetic products needed for plants growth and development. In addition, the differences in plant weight could also be attributed to genetic effect of individual varieties.

One of the important considerations in GM crops 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 important to determine whether the newly introduced protein is significantly similar to any of the known allergen(s) or toxin(s). The Codex Alimentarius Criteria of WHO (Codex, 2003) requires that for a protein to show potential allergenic cross-reactivity, there must be at least 35% sequence identity with an 80 or 8 or 6 amino acid segment between the Query protein (in this case, Cry1Ab) and any of the allergenic or toxic protein. This criteria was not met. Comparison of the primary amino acid sequence and overall structure of the introduced protein with that of the known allergens is an important preliminary step in the safety assessment process. Proteins that are found to be highly similar in sequence or predicted conformational structure to known allergens are then further tested with sera from individuals having allergies to the identified allergen to evaluate possible IgE binding as an indication of their allergenicity. Findings from this study indicated that the percentage Maximum Identity Score (%MIS) and E-score threshold for suspecting cross reactivity was not

met in all the 16 databases used. The inserted protein in the transgenic cowpea resistant against *maruca* virus was in no way classified as allergens nor toxins. Astwood and Fuchs (2000) have described bioinformatics tools as well suited for use in the assessment of potential allergenicity. The results of AllergenOnline version 17.0 database and $BLAST_P$ searches revealed that none of the proteins met the criteria for suspecting allergenic cross-reactivity. The MIS as depicted by FASTA3 search results did not fulfil the criteria for cross-reactivity described by Aalberse (2000) that a protein sharing more than 70% MIS over its length, relative to an allergen is likely to be cross-reactive, or share IgE binding; and those having less than 50% identity are not very likely to be cross-reactive. Furthermore, the presence of any other similar domains in the allergens and Cry proteins were also tested. The search revealed no similarities at the domain level. In a study carried out by Cao *et al.* (2010), an 80 amino acid windows search was done with MIS greater than 35% between the sequences deposited and the Cry8Ka5 and Cry1Ac sequences taken into account. No similarities were detected. The similar results were obtained for the *Cry1C* (Cao *et al.,* 2010) and *Cry1Ab/Ac* proteins (Xu *et al.,* 2009). The importance and validity of MIS in predicting the allergenicity or toxicity of a novel or existing protein may also be seen in one of the previous studies of Mishra *et al.* (2012) where the investigation of the allergenic potential of novel candidate proteins for the development of transgenic was done. The detected proteins with high MIS (37.5 to 97.5%) between the sequences of six known allergens were confirm to be allergenic in animal model. Results from this study is useful in putting an end to the uncertainty and unnecessary fears that have publicly aroused in the mind of many Nigerians especially the anti GMOs.

CHAPTER SIX

6.0 SUMMARY, CONCLUSSION AND RECOMMENDATIONS

6.1 Summary

The major findings of this work can be summarized as follows:

No transgenic events were found in grains of Northern Nigeria markets as at when this research was conducted. Bioinformatics analyses were performed for the recombinant proteins expressed in transgenic cowpeas that are under confined field trials (CFT) in Nigeria. Following morphological comparison between the transgenic and non-transgenic cowpea, no differences was found. The results from the search tools employed in the present study indicates that none of the proteins were positive for potentially allergenic cross-reactivity and toxicity. Hence, the criteria for potential cross-reactivity have not been reached.

The results of the 17 databases used in this studies and $BLAST_P$ searches revealed that none of the proteins met the criteria for suspecting the allergenic cross-reactivity. The percent identity as depicted by FASTA3 search results did not fulfil the criteria for cross-reactivity described by Aalberse (2000) that a protein sharing more than 70% identity over its length, relative to an allergen is likely to be cross-reactive, or share IgE binding; and those having less than 50% identity are not very likely to be cross-reactive. Furthermore, the three domain Cry proteins search also revealed no similarities at the domain level.

6.2 Conclusion

Based on the outcome of this study, the presence of *Cry1Ab, CP4 EPSPS, Bt1Ac, Cry1Fa2 and Pat* events in maize and soybean found in Northern Nigerian markets does not exist as at when this research was conducted.

The molecular characterization of transgenic cowpea carried out in the study confirms the presence of *cry1Ab* gene.

The morphological traits including the seed (colour, size and shape) and leaf (colour, size, pattern and shape) are the same, indicating that the gene inserted into the transgenic cowpea did not alter any of the plants morphological features.

Also the *in silico* risk assessment of the transgenic cowpea confirmed that the event expressed by this plant had no potential of any cross reactivity, toxicity or allergenicity in human.

Thus, it is concluded that the transgenic cowpea resistant against *marucca* may be safe for consumption by both plants and animals.

6.3 Recommendations

Following the findings from this research, it can be recommended that:

Accurate data on the status of GM crops in the Southern and Eastern region of this country (Nigeria) should be done.

Further analysis should be carried out on major shopping malls and supermarkets to ensure the accurate labeling of existing GM crops.

Also, survey for the presence of other events should also be carried out throughout the country.

Government should put an end to the fear in the public about the illegal introduction of GM seeds in the commercial markets of Northern Nigeria by properly educating them with scientific data such as that obtained in this study.

The National Biosafety Management Agency (NBMA) should be strengthened with more adequate facilities to ensure the regulations of transgenic crops in Nigeria.

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APPENDICES

Appendix 1: List of some other GM crops and examples of modified traits

Table 1: List of some GM crops and examples of modified traits

GMO	Traits
Soybean	Increased oleic acid and Stearidonic acid production (David and Siobhan, 2015).
Maize	Increased lysine, Drought tolerance (David and Siobhan, 2015)
Canola	High laurate canola, Phytase production, Delayed senescence (David and Siobhan, 2015)
Potato	Virus resistance, Modified starch (Press, 2010)
Sugar Cane	Drought tolerance (ISAAA, 2016)
Tomato	Delayed softening (ISAAA, 2016)
Tobacco	Herbicide resistance (Tao and Shudongm, 2003)

Appendix 2: Some GM events of Maize and the traits the express

Table 2: Some GM events of Maize and the traits the express

Event Name	Gene Introduced	Method of Introduction	GM Traits
5307	*Pmi, ecry3.1Ab.*	*Agrobacterium tumefaciens*-mpt	Multiple insect resistance, Mannose metabolism (Burns and Raybould, 2014)
*Bt*11 (X4334CBR, X4734CBR)	*Pat, cry1Ab*	Microparticle bombardment	Herbicide tolerance and insect resistance (Takabatake *et al.*, 2013)
*Bt*176 (176)	*cry1Ab, bla, bar*	Microparticle bombardment	Herbicide tolerance, insect and Antibiotic resistance (Song *et al*., 2014)
GA21	*Mepsps*	Microparticle bombardment	Glyphosate herbicide tolerance (Takabatake *et al.*, 2013)
LY038	*cordapA*	Microparticle bombardment	Modified amino acid (Mano *et al.*, 2013)
MIR162	*vip3Aa20, pmi*	*Agrobacterium tumefaciens*-mpt	Insect resistance, Mannose metabolism (Signorini et al., 2018)
MON863	*cry3Bb1, nptII*	Microparticle bombardment	Coleopteran insect and antibiotic resistance (Huang *et*

			al., 2015)
MON89034 x MON88017	*cp4 epsps, cry1A.105, cry2Ab2, cry3Bb1*	Conventional breeding	Herbicide Tolerance + Insect Resistance (Schuppener et al., 2012)
TC1507 x 59122	*cry1F, Pat, cry34Ab1, cry35Ab1*	Conventional breeding	Herbicide Tolerance + Insect Resistance (Lundry *et al.,* 2013)

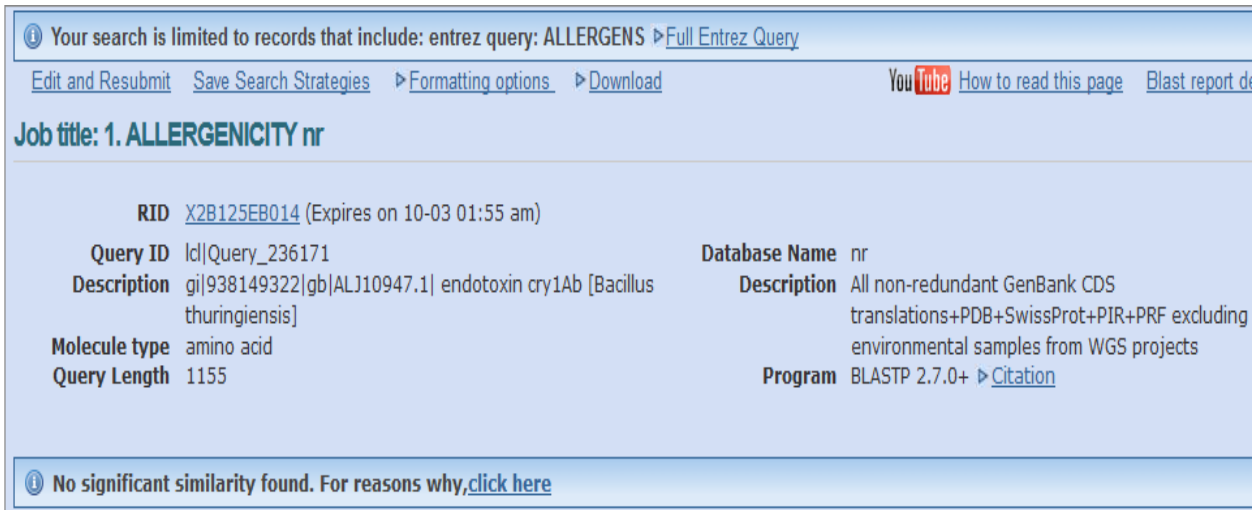
Appendix 3: Some GM events of Soybean and the traits they expressed

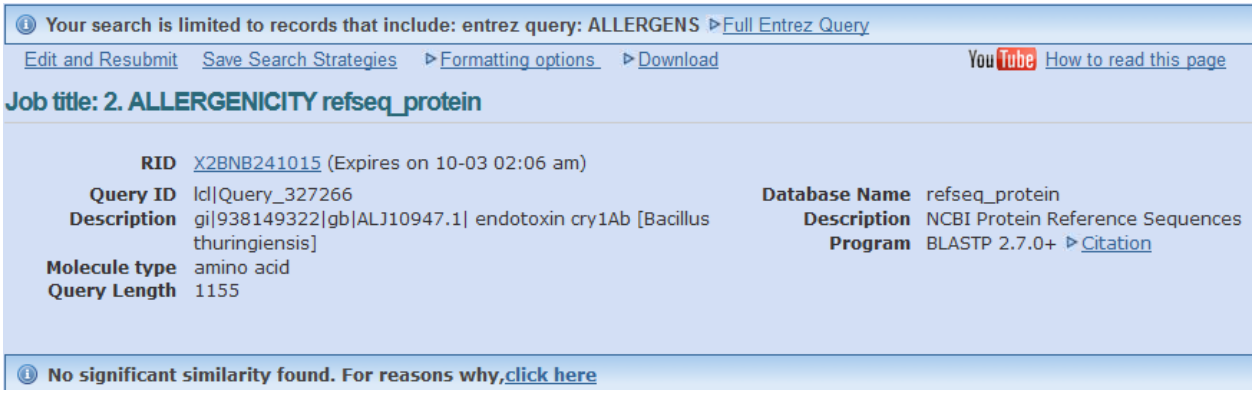
Table 3: Some GM events of Soybean and the traits they expressed

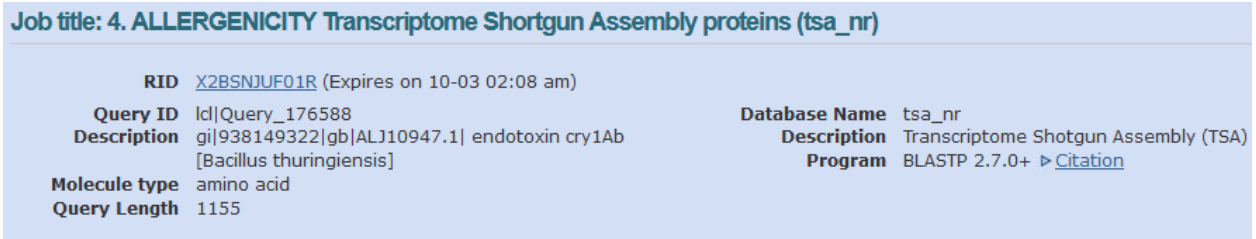
Event name	Genes Introduced	Methods of introduction	Traits introduced
A2704-12	*Pat*	M. bombardment	Herbicide Tolerance (Pi *et al*., 2015)
CV127	*csr1-2*	M. bombardment	Sulfonylurea herbicide tolerance (Waiblinger, 2010)
DAS68416-4 x MON89788	*aad-12, cp4 epsps*	Conventional breeding	Herbicide Tolerance (Sabrina *et al*., 2016)
DAS81419-2	*cry1Ac, cry1F, pat*	*A. tumefaciens*	Insect Resistance (Fast *et al*., 2015)
DP356043	*hra, gat4601*	M. bombardment	Herbicide Tolerance (Pi *et al*., 2015)
GTS 40-3-2 (40-3-2)	*cp4 epsps*	M. bombardment	Herbicide Tolerance (Lievens, 2010)
MON87751	*cry1A.105, cry2Ab2*	*Agrobacterium tumefaciens*	Lepidopteran insect resistance (EURL, 2016)
MON87712	*cp4 epsps, bbx32*	*Agrobacterium tumefaciens*	Glyphosate herbicide tolerance, Enhanced Photosynthesis/Yield (Lutz, 2016)
W62	*Bar*	M. bombardment	Glufosinate herbicide tolerance (Lutz *et al.,* 2016)

A: Agrobacterium: M; Microparticle

Appendix 4: Toxicity and Allergenicity *In silico* Assessment of Cry1Ab Protein using NCBI BLAST$_P$ Omics Tool







Appendix 5: PAN Pesticide Database (PPD) Toxicity information for *Bacillus thuringiensis* Cry1Ab protein

Appendix 5a: PAN Regulatory information

Regulatory Information for Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL004 or pTDL008) in Event T303-3

International Regulatory Status	This Chemical	Parent Chemical
UNEP Persistent Organic Pollutant (POP)	Not Listed	Not Listed
UNEP Prior Informed Consent Chemical (PIC)	Not Listed	Not Listed
WHO Obsolete Pesticide	Not Listed	Not Listed
U.S. and California Regulatory Status		
U.S. EPA Registered	No	No
U.S. EPA Hazardous Air Pollutant	Not Listed	Not Listed
U.S. EPA Minimum Risk Pesticide (25b list)	No	No
CA Registered	No	No
CA Groundwater Contaminant	Not Listed	Not Listed
CA Toxic Air Contaminant	Not Listed	Not Listed

Appendix 5b: PAN Chemical summary

Chemical Summary for Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL004 or pTDL008) in Event T303-3 from www.Pesticideinfo.org

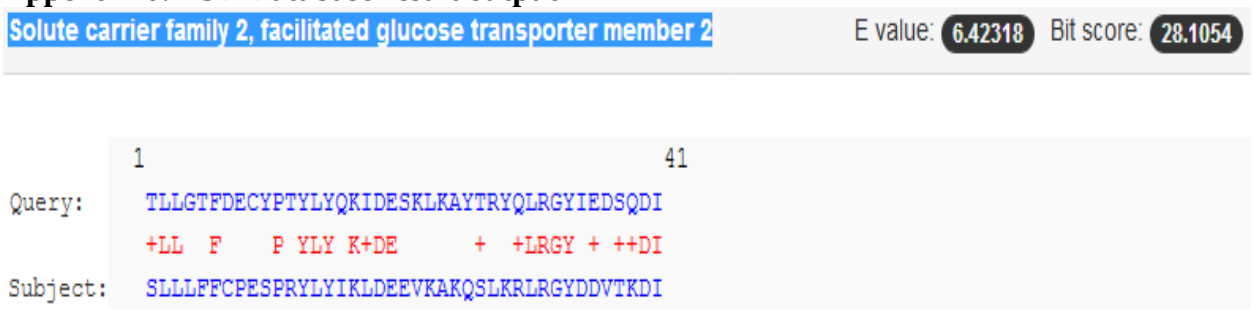
Home > Chemical Search Help

Toxicity of Bacillus thuringiensis Cry1Ab protein and the genetic material necessary for its production (pTDL004 or pTDL008) in Event T303-3

Toxicity Summary by Category

PAN Bad Actor	Acute Toxicity	Carcinogen	Cholinesterase Inhibitor	Water Contaminant	Developmental or Reproductive Toxin	Endocrine Disruptor
Not Listed	?	?	No	?	?	?

Appendix 6: T3D Database result output



Appendix 7: Some selected output for the Full Sequence identity search of Cry1Ab protein

i. Full Sequence identity search using FASTA 35.0 Algorithm of AllergenOnline Database

```
>>gi|167472845|gid|410|pollen allergen Car b 1 isoform [Carpinus betulus]      (160 aa)
 initn:  41 init1:  41 opt:  87  Z-score: 101.8  bits: 27.9 E():  1.5
Smith-Waterman score: 87; 26.5% identity (58.2% similar) in 98 aa overlap (952-1041:54-144)

              920       930       940       950       960       970       980
gi|938 SIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNHRSVLVVPEWEA--
                                                :::  : .:.    :: .::  .. : .   .: : ..
gi|167 IPAARLFKSYVLDGDKLIPKVAPQAISSVENVGGNGGPGTIKNITFAEGIPFKFVKERVDEVDNANFKYSYTVIEGDVLG
             20        30        40        50        60        70        80        90

          990       1000      1010      1020      1030      1040      1050      1060
gi|938 ----EVSQEVRVC--PGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTY
           .:::.:...   :: : :.....  .. :.     ::.  :..:.: .. . :..
gi|167 DKLEKVSHELKIVAAPGGGSIVKISSKFHAKGD-----HEV--NAEEMKGAKEMAEKLLRAVESYLLAHTAEYN
             100       110       120               130       140       150       160

          1070      1080      1090      1100      1110      1120      1130      1140
gi|938 TSRNRGYDGAYESNSSVPADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETEGTF
```

ii. Full sequence identity search using the Allerbase Bioinformatics omics tool

```
> sp|O82015|LIGV1_LIGVU Major pollen allergen Lig v 1 OS=Ligustrum
vulgare PE=1 SV=2
Length=145

 Score = 26.6 bits (57),  Expect = 2.7, Method: Compositional matrix adjust.
 Identities = 28/125 (22%), Positives = 49/125 (39%), Gaps = 12/125 (10%)

Query  47   LSEFVPGAGFVLGLVDIIWGIFGPSQW-DAFLVQIEQLINQRIEEFARNQAISRLEGLSN  105
            LSEF+PGAG   L   D   G  G   + +    + E L N  IE   +N+
Sbjct  30   LSEFIPGAGVRLQCKD---GENGKVTFTEVGYTKAEGLYNMLIERDHKNEFCE-------  79

Query  106  LYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAA  165
               + + S ++ +   PT    ++   ++N +N     T   PL   ++    +P
Sbjct  80   -ITLISSSRKDCDEIPTEGWVKPSLKFVLNTVNGTTRTINPLGFLKKEVLPKCPQVFNKL  138

Query  166  NLHLP  170
             ++ P
Sbjct  139  GMYPP  143
```

iii. Sequence Homology Full FASTA Alignment based on FAO/WHO Allergenicity Rules using SDAP Omics tool

```
Sequence 1: Cry1Ab
Sequence 2: Allergen Pha a 1, Sequence: Q41260
Sequence  1  ----------------------------GSAQGI--------------------
Sequence  2  MMKMVCSSSSSSLLVVAALLAVFVGSAQGIAKVPPGPNITAEYGDKWLDA

Sequence  1  ------------------------------------------------------
Sequence  2  KSTWYGKPTGAGPKDNGGACGYKDVDKAPFNGMTGCGNTPIFKDGRGCGS

Sequence  1  ------------------------------------------------------
Sequence  2  CFELKCSKPESCSGEPITVHITDDNEEPIAPYHFDLSGHAFGSMAKKGEE

Sequence  1  ------------------------------------------------------
Sequence  2  ENVRGAGELELQFRRVKCKYPDGTKPTFHVEKGSNPNYLALLVKYVDGDG

Sequence  1  ------------------------------------------------------
```

Appendix 8: Some selected Windows 80mer search output

i. Windows 80mer search using AllergenOnline Database

80mer Sliding Window Search Results

Database	AllergenOnline Database v17 (January 18, 2017)
Input Query	>gi|938149322|gb|ALJ10947.1| endotoxin cry1Ab [Bacillus thuringiensis] MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLPVLRDVSVFGQ RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV PPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKST NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTS IDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVECLSDEFC LDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKENYV TLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDIEIYLIRYNAKHETVNVPGT GSLWPLSAPSPIGKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFL EEKPLVGEALARVKRAEKKWRGKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNI AMIHAADKRVHSIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNG LSCWNVKGHVDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVT IHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSV PADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETE GTFIVDSVELLLMEE
Length	1155
Number of 80 mers	1076
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

ii. 80mer sequence identity search of Cry1Ab using SDAP 80 amino acids sliding window

Alignment 61
Sequence 1: Cry1Ab; Amino acids 61 - 140
Sequence 2: Allergen Lig v 1, Sequence: Q82015
Sequence identity: 25.00%; Identical amino acids: 20 over a window of 80 amino acids

```
Sequence  1  NPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGLVDIIWGI
             --------------G----D----------LSEF-PGAG--L---D---G-
Sequence  2  EDVPQPPVSQFYIQGQVYCDTCRARFITELSEFIPGAGVRLQCKD---GE

Sequence  1  FGPSQW-DAFLVQIEQLINQRIEEFARNQAI-------------------
             -G------------E-L-N--IE----N---------------------
Sequence  2  NGKVTFTEVGYTKAEGLYNMLIERDHKNEFCEITLISSSRKDCDEIPTEG

Sequence  1  ------------------------------------------------
             ------------------------------------------------
Sequence  2  WVKPSLKFVLNTVNGTTRTINPLGFLKKEVLPKCPQVFNKLGMYPPNM
```

iii. Allermatch database Sequence identity search of Cry1Ab protein

wi_Cor_a_1_b	WHO-IUIS	Cor a 1.0102	UniProt Q08407	Corylus avellana	hazelnut	"natural variant Cor a I/6 with variations at position 81 (T ? K) and 101 (H ? S) compared to accession sequence; initial methionine (1 aa) removed"	159 aa

initn: 83 init1: 55 opt: 85 Z-score: 92.8 bits: 26.2 E(1080): 2.5
Smith-Waterman score: 85; 27.6% identity (56.1% similar) in 98 aa overlap (952-1041:53-143)

Appendix 9: Six amino acid exact word match Homology Alignment based on FAO/WHO Allergenicity Rules using SDAP Omics tool

Alignment 1
Sequence 1: Cry1Ab
Sequence 2: Allergen Blo t 1.0201, Sequence: AAQ24541

```
Sequence  1 --------------------------------------------------
Sequence  2 MKFLLVAALCALVAIGSCKPTREEIKTFEQFKKVFGKVYRNAEEEARREH

Sequence  1 --------------------------------------------------
Sequence  2 HFKEQLKWVEEHNGIDGVEYAINEYSDMSEQEFSFHLSGGGLNFTYMKME

Sequence  1 --------------------------------------------------
Sequence  2 AAKEPLINTYGSLPQNFDWRQKARLTRIRQQGSCGSCWAFAAAGVAESLY

Sequence  1 --------------------------------------------------
Sequence  2 SIQKQQSIELSEQELVDCTYNRYDSSYQCNGCGSGYSTEAFKYMIRTGLV

Sequence  1 --------------------------------------------------
Sequence  2 EEENYPYNMRTQWCNPDVEGQRYHVSGYQQLRYQSSDEDVMYTIQQHGPV

Sequence  1 ----------------------------YTDHAV----------------
Sequence  2 VIYMHGSNNYFRNLGNGVLRGVAYNDAYTDHAVILVGWGTVQGVDYWIIR

Sequence  1 ---------------------------------
Sequence  2 NSWGTGWGNGGYGYVERGHNSLGINNFVTYATL
```

Sequence 1: Cry1Ab
Sequence 2: Allergen Pha a 1, Sequence: Q41260

```
Sequence  1 ------------------------GSAQGI--------------------
Sequence  2 MMKMVCSSSSSSLLVVAALLAVFVGSAQGIAKVPPGPNITAEYGDKWLDA

Sequence  1 --------------------------------------------------
Sequence  2 KSTWYGKPTGAGPKDNGGACGYKDVDKAPFNGMTGCGNTPIFKDGRGCGS

Sequence  1 --------------------------------------------------
Sequence  2 CFELKCSKPESCSGEPITVHITDDNEEPIAPYHFDLSGHAFGSMAKKGEE

Sequence  1 --------------------------------------------------
Sequence  2 ENVRGAGELELQFRRVKCKYPDGTKPTFHVEKGSNPNYLALLVKYVDGDG

Sequence  1 --------------------------------------------------
Sequence  2 DVVAVDIKEKGKDKWIELKESWGAIWRIDTPDKLTGPFTVRYTTEGGTKA

Sequence  1 --------------------
Sequence  2 EFEDVIPEGWKADTHDASK
```

Alignment 2
Sequence 1: Cry1Ab
Sequence 2: Allergen Sal k 1.0302, Sequence: AAX11261

```
Sequence  1 --------------------------------------------------
Sequence  2 QPIPPNPAELESWFQGAVKPVSEQKGLEPSVVQAESGGVETIEVRQDGSG

Sequence  1 --------------------------------------------------
Sequence  2 KFKTISDAVKHVKVGNTKRVIITIGPGEYREKVKIEGLHPYITLYGIDPK
```

Appendix 10: Cry1Ab Gene Sequence

5´-GTCCTTGGTCCGGTAGAGATAGGGACGGATTATAAGATCCAATGTTATCTGAGTC

AAATATTTCCAATCAACTGCGCGCATCCGAGAAAAACAGTGAAAAACAAATGTATC

GGGGTTAACCGTTGAGTAACGAAGCCAATTTAGTTACTACTCCGTTTGTCCGCCTTCT

GGACCGCCACTTAGGCCGT-3´