

**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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**SEPTEMBER, 2018**

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FACULTY OF LIFE SCIENCES,  
AHMADU BELLO UNIVERSITY,  
ZARIA, NIGERIA**

**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.

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Name

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Signature

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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 *BtIAC* test strips of lot number 6M1053, *CryIAC* 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<sub>P</sub> 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 *CryIAb* 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

<i>AAD-12</i>	Aryloxyalkanoate di-oxygenase 12 protein
<i>amy797E</i>	A chimeric thermotolerant alpha-amylase <i>gene</i>
<i>avhppd-03</i>	P-hydroxyphenylpyruvate dioxygenase
<i>bar</i>	A gene which confers resistance to the herbicide
<i>BBX32</i>	An Arabidopsis B-Box Protein bialphos
<i>bla</i>	Beta lactamase enzyme
BMA	British Medical Association
<i>Bt</i>	<i>Bacillus thurigiensis</i>
CFT	Confined Field Trial
<i>cordapA</i>	Gene that codes for dihydrodipicolinate synthase
<i>CP4</i>	<i>Agrobacterium tumefaciens</i> strain
Crispr	Clustered Regularly Interspaced Short
<i>CryIAb</i>	Pesticidal crystal protein
DNA	Deoxyribonucleic Acid
ERA	Environmental Right Association
<i>FATB1</i>	Fatty acyl-ACP thioesterase B gene
GMO	Genetically Modified Organisms
HOSU	History of Safe Use
<i>hppdPF W36</i>	Modified p-hydroxyphenylpyruvate
<i>hra</i>	Heat-resistant agglutinin ( <i>hra</i> ) <i>gene</i> fragment
<i>in silico</i>	In computer simulation
<i>mEPSP</i>	5-enolpyruvylshikimate-3-phosphate modified

<i>ms45</i> .....	Homozygous recessive male sterile construct
<i>Nc.Fad3</i> .....	<i>Neurospora crassa</i> . delta-6 desaturase gene
<i>PAT</i> .....	Phosphinothricin acetyl transferase gene
PCR.....	Polymerase Chain Reaction
<i>Pj.D6D</i> .....	<i>Primula juliae</i> delta 6 desaturase gene
<i>PMI</i> .....	Phosphomannose isomerase gene
rDNA.....	recombinant DNA
RUR HS.....	Roundup Ready Herbicides Resistance Soybean
<i>TALEN</i> .....	Transcription activator-like effector nucleases
<i>uidA</i> .....	Beta-glucuronidase gene
<i>vip3Aa20</i> .....	Vegetative insecticidal protein (vip3Aa variant)
<i>zm-aa1</i> .....	Alpha amylase enzyme gene
<i>zm-hra</i> .....	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. Among the various strategies employed for assessing 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, bioinformatics, molecular genetics and molecular biology are utilized and integrated. Basic GE techniques 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 tolerance 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 commercialised *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 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 its 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 non-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<sub>P</sub> to predict overall similarities at sequence level (ICMR, 2008). While the FASTA and BLAST<sub>P</sub> 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**

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

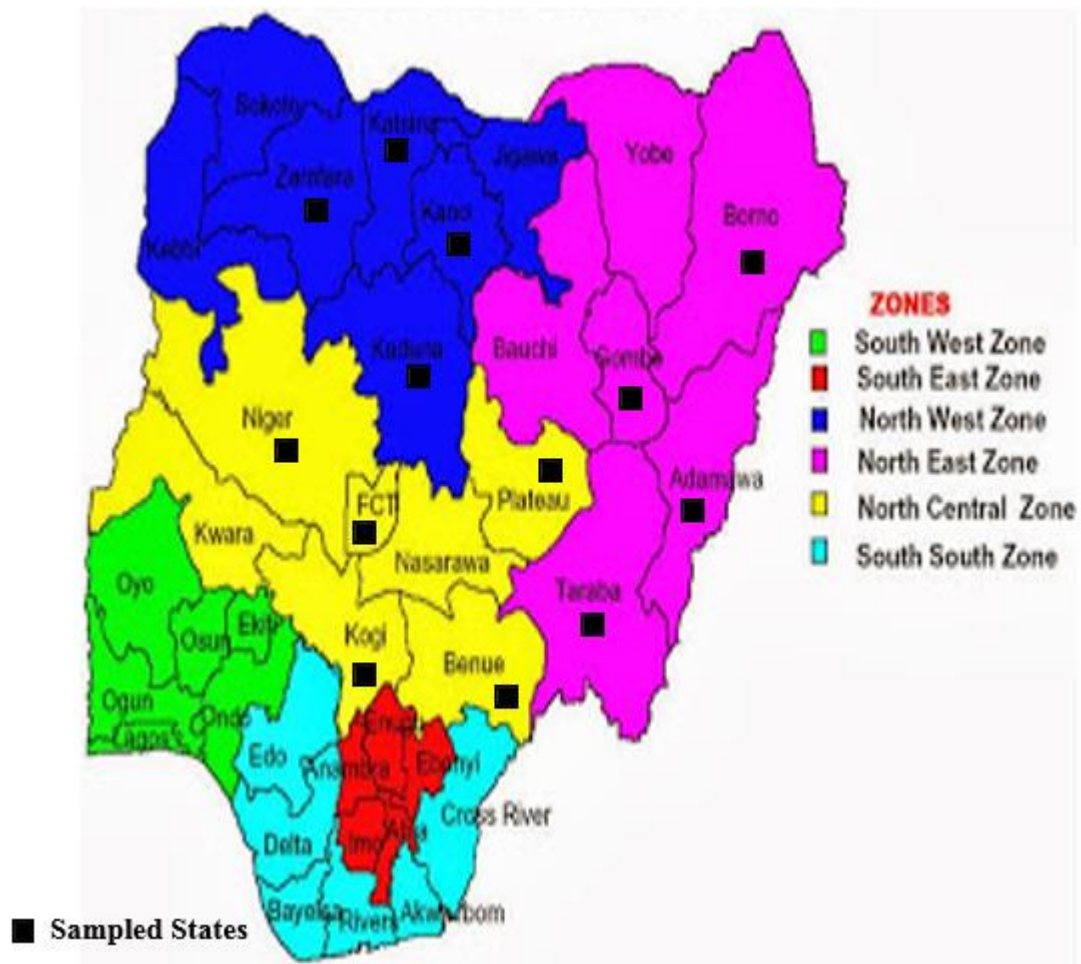


Figure 3.1: The six geopolitical zones of Nigeria. Source: Study and Scholarships (2013).

### 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™ (Romer Labs) based on the manufacturer's recommendation for the detection of *Bt1Ac* (lot number 6M1053), *CryIAc* (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 °C for 10 min, initial denaturation of 30 seconds at 95°C and annealing at 59 °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 MgCl<sub>2</sub>, 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 *CryIAb* gene is presented in table 3.2.

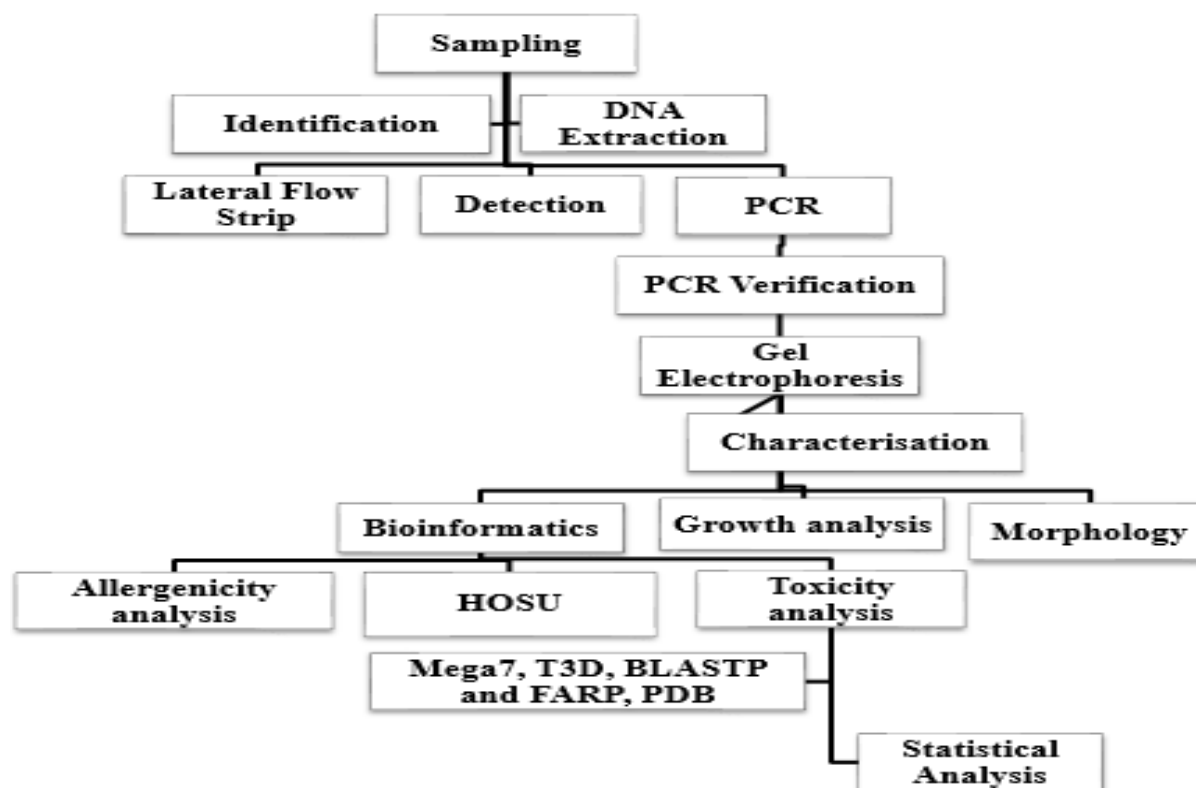


Figure 3.2: Scheme of the experimental procedure for the identification and analysis of transgenic event. *HOSU*: History of Safe Use; *PCR*: Polymerase Chain Reaction; *T3D*: Toxins and Toxin Target Database; *PDB*: Protein Data Bank; *BLASTP*: Protein Basic Alignment Sequence Tool.

**Table 3.2: Selected Oligonucleotide Primers for PCR**

Primer	Sequence (5'-3')	Target Gene	AS (bp)
Forward	5'-CAGGAACCAGGCCATCTCTA-3'	<i>CryIAb</i>	188
Reverse	5'-CGCTCCAAGCCAGTGTGTA-3'	<i>CryIAb</i>	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 CryIab 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  $1 \times 10^{-1}$  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 *CryIAb* gene**

<b>SN</b>	<b>DATABASE</b>	<b>URL</b>
<b>i</b>	Food Allergy Research and Resource program	<a href="http://www.allergenonline.com">www.allergenonline.com</a>
<b>ii</b>	National Centre for Biotechnology Information	<a href="http://www.ncbi.nlm.nih.gov">www.ncbi.nlm.nih.gov</a>
<b>iii</b>	Allergen Families (AllFam)	<a href="http://www.meduniwien.ac.at/allergens/allfam/">www.meduniwien.ac.at/allergens/allfam/</a>
<b>iv</b>	Pan Pesticide Data base (PPD)	<a href="http://www.PesticideInfo.org">www.PesticideInfo.org</a>
<b>v</b>	Interproscan Domain database	<a href="http://www.ebi.ac.uk/Tools/InterProScan/">www.ebi.ac.uk/Tools/InterProScan/</a>
<b>vi</b>	Allergome	<a href="http://www.allergome.org">http://www.allergome.org</a>
<b>vii</b>	Structural Database of Allergenic Proteins	<a href="http://fermi.utmb.edu/SDAP">fermi.utmb.edu/SDAP</a>
<b>viii</b>	Allermatch	<a href="http://www.allermatch.org">http://www.allermatch.org</a>
<b>ix</b>	Pubmed Data Base	<a href="http://www.ncbi.nlm.nih.gov/pubmed/">http://www.ncbi.nlm.nih.gov/pubmed/</a>
<b>x</b>	Allergen Database for Food Safety (ADFS)	<a href="http://allergen.nihs.go.jp/ADFS/">http://allergen.nihs.go.jp/ADFS/</a>
<b>xi</b>	Allerbase	<a href="http://196.1.114.46:1800/AllerBase/HTML/BLAST.html">http://196.1.114.46:1800/AllerBase/HTML/BLAST.html</a>
<b>xii</b>	Universal Protein Resource (UniProt)	<a href="http://www.uniprot.org">http://www.uniprot.org</a>
<b>xiii</b>	IUIS Allergen Nomenclature (IUIS)	<a href="http://www.allergen.org">www.allergen.org</a>
<b>xiv</b>	Food Allergens of Plant Origin (Protall)	<a href="http://www.ifr.bbsrc.ac.uk/Protall">www.ifr.bbsrc.ac.uk/Protall</a>
<b>xv</b>	Toxins and Toxin Target Database (T3D)	<a href="http://www.t3db.ca/biodb/search/target_bonds/sequence">http://www.t3db.ca/biodb/search/target_bonds/sequence</a>
<b>Xvi</b>	WHO-IUIS Database	<a href="http://www.allergen.org">http://www.allergen.org</a>
<b>Xvii</b>	Protein Structure Discovery (PSD)	<a href="http://www-bionet.sccc.ru/psd/">http://www-bionet.sccc.ru/psd/</a>

### 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<sub>P</sub>) 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<sub>P</sub> 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/](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<sub>P</sub> 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<sub>P</sub> 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. *CryIAb* 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  $1 \times 10^{-25}$ ) 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  $\pm$  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/IAc* 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 appropriate *bt* strips also confirmed the absence of the foreign gene, *Cry1Ab*, *Pat*, and *Cry2A/IAc* 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/IAc genes* all gave no band. Similar analysis carried out for other genes also gave the same result.

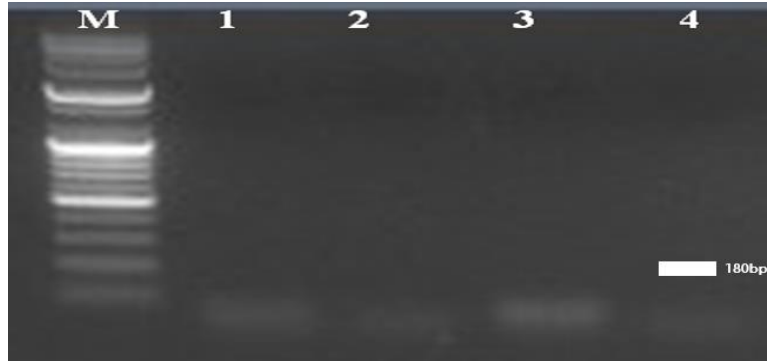
A total of 12 Northern Nigeria states were surveyed from between March, 2016 to June, 2017. The result of the survey is presented in table 4.1 (a, b and c). The summary of the flow strip test and PCR detection results shows a negative result for all sampled locations.



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 *CryIAb*.** 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/IAc*.** 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
		<i>PAT</i>	<i>Bt1Ac</i>	<i>Cry1ac</i>	<i>Cry2a</i>	
<b>Abuja</b>	au1	-	-	-	-	Absent
	au2	-	-	-	-	Absent
	au3	-	-	-	-	Absent
	au4	-	-	-	-	Absent
	au5	-	-	-	-	Absent
	au6	-	-	-	-	Absent
	au7	-	-	-	-	Absent
	au8	-	-	-	-	Absent
	au9	-	-	-	-	Absent
<b>Benue State</b>	bn1	-	-	-	-	Absent
	bn2	-	-	-	-	Absent
	bn3	-	-	-	-	Absent
	bn4	-	-	-	-	Absent
	bn5	-	-	-	-	Absent
	bn6	-	-	-	-	Absent
	bn7	-	-	-	-	Absent
	bn8	-	-	-	-	Absent
	bn9	-	-	-	-	Absent
<b>Kogi State</b>	kg1	-	-	-	-	Absent
	kg2	-	-	-	-	Absent
	kg3	-	-	-	-	Absent
	kg4	-	-	-	-	Absent
	kg5	-	-	-	-	Absent
	kg6	-	-	-	-	Absent
	kg7	-	-	-	-	Absent
	kg8	-	-	-	-	Absent
	kg9	-	-	-	-	Absent
<b>Niger State</b>	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
		<i>PAT</i>	<i>Bt1Ac</i>	<i>Cry1ac</i>	<i>Cry2a</i>	
<b>Adamawa State</b>	aa1	-	-	-	-	Absent
	aa2	-	-	-	-	Absent
	aa3	-	-	-	-	Absent
	aa4	-	-	-	-	Absent
	aa5	-	-	-	-	Absent
	aa6	-	-	-	-	Absent
	aa7	-	-	-	-	Absent
	aa8	-	-	-	-	Absent
	aa9	-	-	-	-	Absent
<b>Borno State</b>	br1	-	-	-	-	Absent
	br2	-	-	-	-	Absent
	br3	-	-	-	-	Absent
	br4	-	-	-	-	Absent
	br5	-	-	-	-	Absent
	br6	-	-	-	-	Absent
	br7	-	-	-	-	Absent
	br8	-	-	-	-	Absent
	br9	-	-	-	-	Absent
<b>Gombe State</b>	gm1	-	-	-	-	Absent
	gm2	-	-	-	-	Absent
	gm3	-	-	-	-	Absent
	gm4	-	-	-	-	Absent
	gm5	-	-	-	-	Absent
	gm6	-	-	-	-	Absent
	gm7	-	-	-	-	Absent
	gm8	-	-	-	-	Absent
	gm9	-	-	-	-	Absent
<b>Taraba State</b>	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
		<i>PAT</i>	<i>Bt1Ac</i>	<i>Cry1ac</i>	<i>Cry2a</i>	
<b>Kaduna State</b>	kd1	-	-	-	-	Absent
	kd2	-	-	-	-	Absent
	kd3	-	-	-	-	Absent
	kd4	-	-	-	-	Absent
	kd5	-	-	-	-	Absent
	kd6	-	-	-	-	Absent
	kd7	-	-	-	-	Absent
	kd8	-	-	-	-	Absent
	kd9	-	-	-	-	Absent
<b>Katsina State</b>	kt1	-	-	-	-	Absent
	kt2	-	-	-	-	Absent
	kt3	-	-	-	-	Absent
	kt4	-	-	-	-	Absent
	kt5	-	-	-	-	Absent
	kt6	-	-	-	-	Absent
	kt7	-	-	-	-	Absent
	kt8	-	-	-	-	Absent
	kt9	-	-	-	-	Absent
<b>Kano State</b>	kn1	-	-	-	-	Absent
	kn2	-	-	-	-	Absent
	kn3	-	-	-	-	Absent
	kn4	-	-	-	-	Absent
	kn5	-	-	-	-	Absent
	kn6	-	-	-	-	Absent
	kn7	-	-	-	-	Absent
	kn8	-	-	-	-	Absent
	kn9	-	-	-	-	Absent
<b>Zamfara State</b>	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<sub>P</sub> Omics Tool**

The BLAST<sub>P</sub> 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

Reference 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 *CryIAb* gene showing positive results for *maruca* resistant transgenic cowpea and negative for the non-transgenic cowpea line.

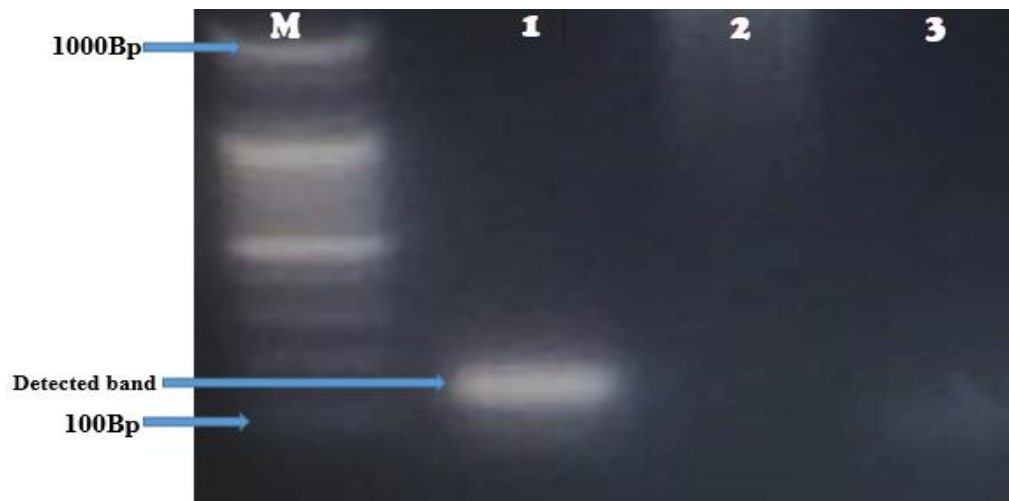


Plate IV: Gel photo of PCR products of *cryIAb* gene from cowpea sample. Lane M: 1000 Bp marker; Lane 2: transgenic cowpea; Lane 3: ddH2O; Lane 4: conventional cowpea.

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

<b>Cry Protein</b>	<b>Result</b>	<b>Findings</b>	<b>Reference</b>
<i>CryIAb</i>	NSD	CryIAb proteins are safe for the lacewing, <i>C. Pallens</i>	Ali <i>et al.</i> , 2017
<i>CryIAc/2Ab</i>	NSD	No detrimental effects on adult honey bee	Niu <i>et al.</i> , 2017
<i>CryIAc/2Ab</i>	CD	Negligible exposure of phloem sucking hemipterans	Meissle, 2017
<i>CryIAb/2Aj</i>	r <sub>m</sub>	No lethal or sub lethal effects	Zhang <i>et al.</i> , 2017
NS	NSD	No adverse effect on the MCC	Liu <i>et al.</i> , 2017
NS	NSD	No significant effects on soil health	Zhaolei <i>et al.</i> , 2017
<i>CryIAb</i>	NSD	Stable aphid population density in <i>Bt</i> rice fields.	Ren <i>et al.</i> , 2016
<i>IAb/IAc</i>	RB	Rapid degradation of the cry protein found.	Liu <i>et al.</i> , 2016
<i>IAb/IAc</i>	NSD	GM rice is equivalent to its parental rice line MH63	Mao <i>et al.</i> , 2016
<i>CryIIe</i>	NSD	No adverse effect on midgut bacteria diversity	Jia <i>et al.</i> , 2016
<i>CryIAc</i>	HLE	High dose criterion met up to 50 times.	Dourado <i>et al.</i> , 2016
NS	Safe	No significant harmful impact has been reported.	Yaqoob <i>et al.</i> , 2016
NS	NSD	Cry proteins are unlikely to function as adjuvants	Joshi <i>et al.</i> , 2016
<i>CryIAc</i>	NSD	No adverse impacts of <i>Bt</i> on species abundance, diversity and community dynamics.	Navasero <i>et al.</i> , 2016
<i>CryIAb/IAc</i>	NSD	Frog development was not affected by dietary intake	Zhu <i>et al.</i> , 2015
<i>CryIAb/IAc</i>	NSD	Safe for use as feed and food	Li <i>et al.</i> , 2015
<i>CryIAc</i>	RB	No detected Cry protein in the soil surrounding	Xiao <i>et al.</i> , 2015
<i>CryIAc</i>	NSD	No adverse effects in RS of male rats	Guo <i>et al.</i> , 2015
Cry2Aa	NSD	No detrimental effects on <i>C. lividipennis</i>	Han <i>et al.</i> , 2014
<i>CryIAh</i>	NSD	No adverse immunotoxicological effects of GM corn	Song <i>et al.</i> , 2014
<i>CryIAb</i>	NSD	Has no significant long-term (90 day) toxic effects	Wang <i>et al.</i> , 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**

<b>Cry Protein</b>	<b>Result</b>	<b>Findings</b>	<b>Reference</b>
<i>IAc/Aa/ICa</i>	NSD	<i>Bt</i> rice have no detrimental effects on any of the physiological processes of BPH.	Mannakkara <i>et al.</i> , 2013
<i>CryIAc-M</i>	NSD	<i>BT-38</i> maize is as safe as it conventional maize.	Liu <i>et al.</i> , 2012
<i>Cry3Bb1</i>	NSD	No adverse effects on various NTO	Devos <i>et al.</i> , 2012
<i>Cry1C</i>	NSD	<i>Cry1C</i> protein is not a potential allergen or toxin	Cao <i>et al.</i> , 2010
<i>Cry1Ab/Ac</i>	NSD	Safe for use as feed and food	Xu <i>et al.</i> , 2009
<i>Cry1Ab</i>	NSD	No potential risk of transfer	Guertler <i>et al.</i> , 2009
<i>Cry1Ab</i>	NSD	No toxicity was observed at high concentrations	Bondzio <i>et al.</i> , 2008
<i>NS</i>	NSD	No uniform effects of <i>Bt</i> crops on the functional guilds.	Wolfenbarger <i>et al.</i> , 2008
<i>Cry1Ab</i>	NSD	No adverse effects of <i>Cry1Ab</i> in the 90-day study	Schrøder <i>et al.</i> , 2007
<i>Cry1Ac</i>	NSD	GM grains were equivalent to their non-transgenic	Li <i>et al.</i> , 2007
<i>IAc/2Ab2</i>	NSD	GM cotton is safe for food and use	Hamilton <i>et al.</i> , 2004
<i>Cry1F</i>	NSD	<i>Cry1F</i> maize was equivalent to non-GM maize	Herman <i>et al.</i> , 2004
<i>Cry3Bb1</i>	NSD	MON 863 is safe and nutritious as it non GM corn	George <i>et al.</i> , 2004
<i>Cry1Ab</i>	AS	<i>Bt</i> hybrids can increase the percentage of corn grain that would be suitable for use in food and feed	Hammond <i>et al.</i> , 2004
<i>Cry1Ab</i>	AS	No toxicity was observed	Noteborn <i>et al.</i> , 1994
<i>IAa/IAb</i>	AS	No evidence of toxicity/ infectivity/ pathogenicity	David, 1989
<i>CryIAa/b/c/2A</i>	NSD	Not a virulent or invasive mammalian pathogen.	Hadley <i>et al.</i> , 1987
<i>NS</i>	NSD	Crystal delta endotoxin, produced no pathological effects by oral administration.	Thomas and Ellar, 1983
<i>Cry1Ab</i>	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<sub>P</sub> 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**

<b>Factors</b>	<b>Result</b>	<b>Remark</b>
<b>PAN Bad Actor Chemical</b>	NL	Not Toxic
<b>Acute Toxicity</b>	NWE	Not Toxic
<b>Carcinogen</b>	NWE	Not Toxic
<b>Cholinesterase Inhibitor</b>	NL	Not Toxic
<b>Ground Water Contaminant</b>	NWE	Not Toxic
<b>Developmental or Reproductive Toxin</b>	NWE	Not Toxic
<b>Endocrine Disruptor</b>	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
<b>FARRP<sup>a</sup></b>	<i>Car b 1 PA</i>	ABZ81044.1	26.5	1.5	< 0.02	-	NLA
<b>FARRP<sup>b</sup></b>	<i>Major allergen</i>	CAA50328.1	27.6	2.0	< 0.02	-	NLA
<b>FARRP<sup>c</sup></b>	<i>Pollen allergen</i>	ADK39021.1	27.6	2.0	< 0.02	-	NLA
<b>Allerbase<sup>a</sup></b>	<i>Ligv1_ligvu</i>	O82015	-	2.7	0.01	-	26.6 CNM
<b>Allerbase<sup>c</sup></b>	<i>Pert_human</i>	P07202	-	9.6	0.01	-	25.8 CNM
<b>ADFS</b>	<i>MPA Lig V 1</i>	KHG25921.1	-	22	0.02	-	AWN
<b>Allermatch<sup>a</sup></b>	<i>CYN d 23.0101</i>	AAP80170.1	-	19.5	0.02	-	AWN
<b>Allermatch<sup>b</sup></b>	<i>COR a 1.0102</i>	CAA50327.1	-	27.6	0.02	-	AWN
<b>SDAP<sup>a</sup></b>	Tria a gladin	AAA34285	-	3.62	-	36/995	- CNM
<b>SDAP<sup>b</sup></b>	Mala s 1	Q01940	-	4.12	-	41/995	- CNM
<b>SDAP<sup>c</sup></b>	Pen ch 200101	AAB34785	-	2.01	-	20/995	- CNM
<b>SDAP<sup>d</sup></b>	Bra n 1	P80208	-	2.61	-	26/995	- CNM
<b>SDAP<sup>e</sup></b>	Asp f 5	CAA83015	-	5.83	-	58/995	- CNM
<b>SDAP<sup>f</sup></b>	Car b 1032	CAB02217	-	2.71	-	27/995	- CNM
<b>SDAP<sup>g</sup></b>	Cyn d 23	AAP80170	-	1.71	-	17/995	- CNM

<sup>a,b,c</sup>: 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 <sup>a</sup>	Plaor1.0101	ABY21305	1-80	8	10	>35%	CNM
SDAP <sup>b</sup>	Eurm3	O97370	5 - 84	10	12.5	>35%	CNM
SDAP <sup>c</sup>	Ligv1	O82015	61 - 140	20	25.0	>35%	CNM
SDAP <sup>d</sup>	Alta2	AAD00097	91 - 170	17	21.25	>35%	CNM
SDAP <sup>e</sup>	Bosd8	AAA30478	131 - 210	16	20.0	>35%	CNM
SDAP <sup>f</sup>	Fage1	O9XFM4	150 - 229	12	15	>35%	CNM
SDAP <sup>g</sup>	Gald4	P00698	201 - 280	13	16.25	>35%	CNM
SDAP <sup>h</sup>	Pench20.010	AAB34785	282 - 361	16	20	>35%	CNM
SDAP <sup>i</sup>	Musa2.0101	O8VXF1	317 - 396	21	26.25	>35%	CNM
SDAP <sup>j</sup>	Bran1	P80208	343 - 422	18	22.5	>35%	CNM
SDAP <sup>k</sup>	Hevb1	P15252	415 - 494	22	27.5	>35%	CNM
SDAP <sup>l</sup>	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**

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

**Table 4.7: Six amino acid exact word match search**

<b>Database</b>	<b>Allergen</b>	<b>6-AA EWM</b>	<b>MI (%)</b>	<b>% Cut off</b>	<b>Inference</b>
<b>WHO-IUIS<sup>a</sup></b>	wi_Tri_a_34	1	0.09	35%	NA
<b>WHO-IUIS<sup>b</sup></b>	wi_Sal_k_1_d	1	0.09	35%	NA
<b>WHO-IUIS<sup>c</sup></b>	i_Jun_v_1_b	1	0.09	35%	NA
<b>WHO-IUIS<sup>d</sup></b>	wi_Eur_m_14	1	0.09	35%	NA
<b>UniProt/ WI<sup>a</sup></b>	al_Ph1_p_11	1	0.09	35%	NA
<b>UniProt/ WI<sup>b</sup></b>	al_Lol_p_11	1	0.09	35%	NA
<b>UniProt/ WI<sup>c</sup></b>	al_Jun_v_1_a	1	0.09	35%	NA
<b>UniProt/ WI<sup>d</sup></b>	al_Jun_a_1_b	1	0.09	35%	NA
<b>SDAP<sup>a</sup></b>	Blot1.0201	1	0.9	35%	CNM
<b>SDAP<sup>b</sup></b>	PhAA1	1	0.9	35%	CNM
<b>SDAP<sup>c</sup></b>	CupA1	1	0.9	35%	CNM
<b>SDAP<sup>d</sup></b>	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 IT97KT) 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.

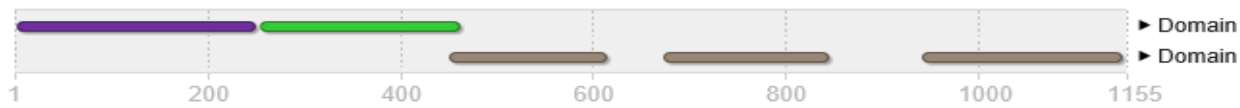
##### **4.7.2 Weight analysis of the three cowpea lines**

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.

## Protein family membership

None predicted.

## Domains and repeats



## Detailed signature matches

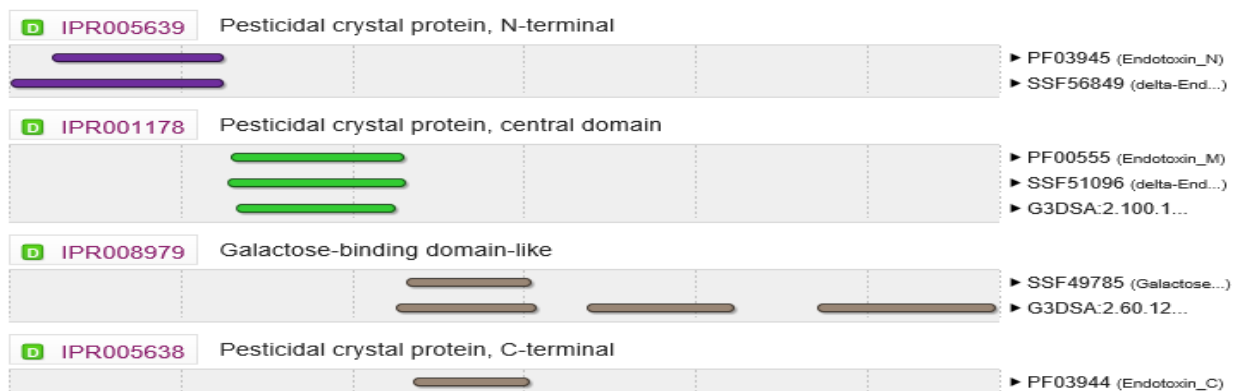


Figure 4.1 b: Detailed signature matches



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

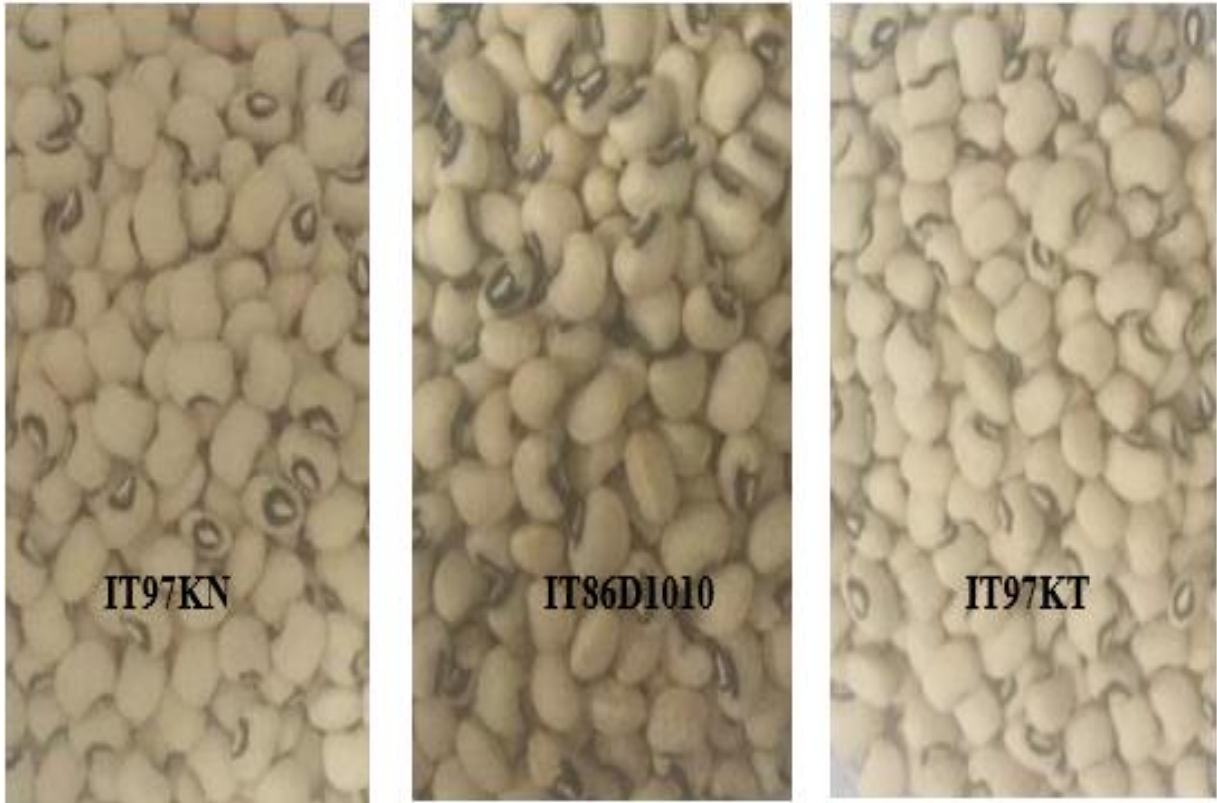


Plate V: Seeds of three different lines of cowpea. Line IT86D1010 and IT97KT are transgenic while line IT97KN is non-transgenic

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.

#### 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.



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 <sup>2</sup> dayG <sup>1</sup> )			
	10 DAP	20 DAP	30 DAP	40 DAP
<b>IT86D1010</b>	161.5000±19.092 <sup>a</sup>	266.5000±9.192 <sup>a</sup>	286.0000±4.243 <sup>a</sup>	285.5000±4.950 <sup>a</sup>
<b>IT97KT</b>	142.0000±16.971 <sup>a</sup>	229.0000±4.243 <sup>b</sup>	250.5000±4.950 <sup>b</sup>	250.0000±5.657 <sup>b</sup>
<b>IT97KN</b>	132.0000±4.243 <sup>a</sup>	213.5000±10.607 <sup>b</sup>	219.0000±12.728 <sup>c</sup>	219.0000±11.314 <sup>c</sup>

*N=2; <sup>abc</sup>\*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 ( $\text{gG}^2 \text{ dayG}^{-1}$ )			
	10 DAP	20 DAP	30 DAP	40 DAP
<b>IT86D1010</b>	5.10±0.10 <sup>a</sup>	11.60±0.20 <sup>a</sup>	9.73±2.81 <sup>a</sup>	12.93±4.21 <sup>a</sup>
<b>IT97KT</b>	2.97±0.06 <sup>b</sup>	8.80±0.10 <sup>a</sup>	9.67±0.40 <sup>a</sup>	13.77±0.31 <sup>a</sup>
<b>IT97KN</b>	2.27±0.06 <sup>c</sup>	26.40±34.73 <sup>a</sup>	7.23±0.59 <sup>a</sup>	9.20±0.30 <sup>a</sup>

N=3; <sup>abc\*</sup>Values within the treatment group in the same column followed by same superscript (s) are not significantly different at ( $p \leq 0.05$ ); DAP: Days After Planting;  $\pm$ : 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 *CryIAb* protein safe and nontoxic. Analysis by HOSU indicates that *CryIAb* 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<sub>P</sub> 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, CONCLUSION 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<sub>P</sub> 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 *cryIAb* 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**

<b>GMO</b>	<b>Traits</b>
<b>Soybean</b>	Increased oleic acid and Stearidonic acid production (David and Siobhan, 2015).
<b>Maize</b>	Increased lysine, Drought tolerance (David and Siobhan, 2015)
<b>Canola</b>	High laurate canola, Phytase production, Delayed senescence (David and Siobhan, 2015)
<b>Potato</b>	Virus resistance, Modified starch (Press, 2010)
<b>Sugar Cane</b>	Drought tolerance (ISAAA, 2016)
<b>Tomato</b>	Delayed softening (ISAAA, 2016)
<b>Tobacco</b>	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	<i>Pmi, ecry3.1Ab.</i>	<i>Agrobacterium tumefaciens</i> -mpt	Multiple insect resistance, Mannose metabolism (Burns and Raybould, 2014)
Bt11 (X4334CBR, X4734CBR)	<i>Pat, cry1Ab</i>	Microparticle bombardment	Herbicide tolerance and insect resistance (Takabatake <i>et al.</i> , 2013)
Bt176 (176)	<i>cry1Ab, bla, bar</i>	Microparticle bombardment	Herbicide tolerance, insect and Antibiotic resistance (Song <i>et al.</i> , 2014)
GA21	<i>Mepsps</i>	Microparticle bombardment	Glyphosate herbicide tolerance (Takabatake <i>et al.</i> , 2013)
LY038	<i>cordapA</i>	Microparticle bombardment	Modified amino acid (Mano <i>et al.</i> , 2013)
MIR162	<i>vip3Aa20, pmi</i>	<i>Agrobacterium tumefaciens</i> -mpt	Insect resistance, Mannose metabolism (Signorini <i>et al.</i> , 2018)
MON863	<i>cry3Bb1, nptII</i>	Microparticle bombardment	Coleopteran insect and antibiotic resistance (Huang <i>et al.</i> , 2015)
MON89034 MON88017	x <i>cp4 epsps, cry1A.105,</i> <i>cry2Ab2, cry3Bb1</i>	Conventional breeding	Herbicide Tolerance + Insect Resistance (Schuppener <i>et al.</i> , 2012)
TC1507 x 59122	<i>cry1F, Pat, cry34Ab1,</i> <i>cry35Ab1</i>	Conventional breeding	Herbicide Tolerance + Insect Resistance (Lundry <i>et al.</i> , 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
<b>A2704-12</b>	<i>Pat</i>	M. bombardment	Herbicide Tolerance (Pi <i>et al.</i> , 2015)
<b>CV127</b>	<i>csr1-2</i>	M. bombardment	Sulfonylurea herbicide tolerance (Waiblinger, 2010)
<b>DAS68416-4 x MON89788</b>	<i>aad-12, cp4 epsps</i>	Conventional breeding	Herbicide Tolerance (Sabrina <i>et al.</i> , 2016)
<b>DAS81419-2</b>	<i>cry1Ac, cry1F, pat</i>	<i>A. tumefaciens</i>	Insect Resistance (Fast <i>et al.</i> , 2015)
<b>DP356043</b>	<i>hra, gat4601</i>	M. bombardment	Herbicide Tolerance (Pi <i>et al.</i> , 2015)
<b>GTS 40-3-2 (40-3-2)</b>	<i>cp4 epsps</i>	M. bombardment	Herbicide Tolerance (Lievens, 2010)
<b>MON87751</b>	<i>cry1A.105, cry2Ab</i>	<i>Agrobacterium tumefaciens</i>	Lepidopteran insect resistance (EURL, 2016)
<b>MON87712</b>	<i>cp4 epsps, bbx32</i>	<i>Agrobacterium tumefaciens</i>	Glyphosate herbicide tolerance, Enhanced Photosynthesis/Yield (Lutz, 2016)
<b>W62</b>	<i>Bar</i>	M. bombardment	Glufosinate herbicide tolerance (Lutz <i>et al.</i> , 2016)

A: Agrobacterium; M; Microparticle

## Appendix 4: Toxicity and Allergenicity *In silico* Assessment of Cry1Ab Protein using NCBI

### BLAST<sub>P</sub> Omics Tool

**Job title: 1. ALLERGENICITY nr**

**RID** [X2B125EB014](#) (Expires on 10-03 01:55 am)

<b>Query ID</b>	ld Query_236171	<b>Database Name</b>	nr
<b>Description</b>	gi 938149322 gb ALJ10947.1  endotoxin cry1Ab [Bacillus thuringiensis]	<b>Description</b>	All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
<b>Molecule type</b>	amino acid	<b>Program</b>	BLASTP 2.7.0+ <a href="#">▶ Citation</a>
<b>Query Length</b>	1155		

**No significant similarity found. For reasons why, [click here](#)**

**Job title: 2. ALLERGENICITY refseq\_protein**

**RID** [X2BNB241015](#) (Expires on 10-03 02:06 am)

<b>Query ID</b>	ld Query_327266	<b>Database Name</b>	refseq_protein
<b>Description</b>	gi 938149322 gb ALJ10947.1  endotoxin cry1Ab [Bacillus thuringiensis]	<b>Description</b>	NCBI Protein Reference Sequences
<b>Molecule type</b>	amino acid	<b>Program</b>	BLASTP 2.7.0+ <a href="#">▶ Citation</a>
<b>Query Length</b>	1155		

**No significant similarity found. For reasons why, [click here](#)**

**Job title: 4. ALLERGENICITY Transcriptome Shotgun Assembly proteins (tsa\_nr)**

**RID** [X2BSNJUF01R](#) (Expires on 10-03 02:08 am)

<b>Query ID</b>	ld Query_176588	<b>Database Name</b>	tsa_nr
<b>Description</b>	gi 938149322 gb ALJ10947.1  endotoxin cry1Ab [Bacillus thuringiensis]	<b>Description</b>	Transcriptome Shotgun Assembly (TSA)
<b>Molecule type</b>	amino acid	<b>Program</b>	BLASTP 2.7.0+ <a href="#">▶ Citation</a>
<b>Query Length</b>	1155		

**No significant similarity found. For reasons why, [click here](#)**

## 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
<a href="#">UNEP Persistent Organic Pollutant (POP)</a>	Not Listed	Not Listed
<a href="#">UNEP Prior Informed Consent Chemical (PIC)</a>	Not Listed	Not Listed
<a href="#">WHO Obsolete Pesticide</a>	Not Listed	Not Listed
<b>U.S. and California Regulatory Status</b>		
<a href="#">U.S. EPA Registered</a>	No	No
<a href="#">U.S. EPA Hazardous Air Pollutant</a>	Not Listed	Not Listed
<a href="#">U.S. EPA Minimum Risk Pesticide (25b list)</a>	No	No
<a href="#">CA Registered</a>	No	No
<a href="#">CA Groundwater Contaminant</a>	Not Listed	Not Listed
<a href="#">CA Toxic Air Contaminant</a>	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](http://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

<a href="#">PAN Bad Actor</a>	<a href="#">Acute Toxicity</a>	<a href="#">Carcinogen</a>	<a href="#">Cholinesterase Inhibitor</a>	<a href="#">Water Contaminant</a>	<a href="#">Developmental or Reproductive Toxin</a>	<a href="#">Endocrine Disruptor</a>
Not Listed	?	?	No	?	?	?

## Appendix 6: T3D Database result output

Solute carrier family 2, facilitated glucose transporter member 2

E value: 6.42318 Bit score: 28.1054

```
1                                     41
Query:  TLLGTFDECYPTYLYQKIDESKPKAYTRYQLRGYIEDSQDI
        +LL F  P YLY K+DE  + +LRGY + ++DI
Subject: SLLLFPCPESPRYLYIKLDEEVKAKQSLKRLRGYDDVTKDI
```

## 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 initl: 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  SIREAYLPPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSQWNVKGVHDVVEEQNNHRSVLVVPWEA--
          :::  :  .:  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
gi|167  IPAARLFKSYVLDGDKLIPKVAPQAISSVENVGGNGGPGTIKNITFAEGIPFKFVKERVDEVDNANFKYSYTVIEGDVLG
          20          30          40          50          60          70          80          90

          990          1000          1010          1020          1030          1040          1050          1060
gi|938  ----EVSQEVVRC--PGRGYILRVTAAYKEGYGEGCVTIHEIENNTDELKFSNCVVEEVYPNNTVTCNDYTATQEEYEGTY
          .:  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
gi|167  DKLEKVSHELKIVAAPGGGSIVKISSKFHAKG-----HEV--NAEEMKGAKEMAELKLLRAVESYLLAHTAEYN
          100          110          120          130          140          150          160

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

### 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 LSEFVPGAGFVLGLVDIIWGFIFGPSQW-DAFLVQIEQLINQRIEEFARNQAISRLEGLSN 105
Sbjct 30 LSEF+PGAG L D G G + + + E L N IE +N+
LSEFIPGAGVRLQCKD---GENGKVTFTFVGYTKAEGLYNMLIERDHKNEFCE----- 79

Query 106 LYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAA 165
Sbjct 80 -ITLISSSRKDCDEIPTEGWVKPSLKFVLTNTVNGTTRTINPLGLKKEVLPKCPQVFNKL 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 1 -----GSAQGI-----
Sequence 2 MMKMVCSSSSSSLLVVAALLAVFVGSQAQGI AKVPPGPNITAEYGDKWLDA

Sequence 1 -----
Sequence 2 KSTWYGKPTGAGPKDNGGACGYKDVDKAPFNGMTGCGNTPIFKDGRGCGS

Sequence 1 -----
Sequence 2 CFELKCSKPESCSGEPITVHITDDNEEPIAPYHFDLSGHAFGSMAKKKEE

Sequence 1 -----
Sequence 2 ENVRGAGELELQFRRVKCKYPDGTPKPTFHVEKGSNPNYLALLVKYVDGDS

Sequence 1 -----
Sequence 2 DVVAVDIKEKGDKWIELKESWGAIWRIDTDPDKLTGPFVRYTTEGGTKA

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

## Appendix 8: Some selected Windows 80mer search output

### i. Windows 80mer search using AllergenOnline Database

#### 80mer Sliding Window Search Results

<b>Database</b>	AllergenOnline Database v17 (January 18, 2017)
<b>Input Query</b>	>gi 938149322 gb ALJ10947.1  endotoxin cry1Ab [Bacillus thuringiensis] MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL VDIIWGI FGPSQWDAFLVQIEQLINQRIEEFARNQAI SRLEGLSNLYQIYAESFREWEAD PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLPVLRDVSVFGQ RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERWVGFDSRDWIRYNQFRRELLTV LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSGFRGSAQGI EGSIRSPHMDIL NSITIIYTD AHRGEYYWSGHQIMASPVGFSGPEFTFFLYGTMGNAAPQQRIVAQLGQGVYR TSSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSQGTVDLDEIPQNNNV PFRQGFSHRLSHVSMFRSGFSNSSVSI IIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKST NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNI TAPLSQRVRYRIRYASTTNLQFHST IDGRPINQGNFSATMSSGSLQSGSFRTVGF TTPFNFSNGSSVFTLSAHVFN SGNVEYID RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKT DVTDYHIDQVSNLVECLSDFEC LDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKENYV TLLGTFDECYPTYLYQKIDESKCLKAYTRYQLRGYIEDSQDIEIYLIRYNAKHETVNVVPGT GSLWPLSAPSPIGKCAHSHHSLDDIDVGC TDLNEDLGVWVIFKIKTQDGHARLGNLEFL EEKPLVGEALARVKRAEKKWRGKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNI AMIHAADKRVHSIREAYLPELSVIPGVNAAI FEELEGRIFTA FSLYDARNVIKNGDFNNG LSCWNVKGHVDVEEQNNHRSVYLVVPEWEAEV SQEVRVCPGRGYILRV TAYKEGEGCVT IHEIENNTDELKFSNVEEYVYVNNVTCTNDYATATQEEYEGTYTSRNRGYD GAYESNSSV PADYASAYEEKAYTDGRRDNPCESNRGYDY TPLPAGYVTKELYFPETDKVWIEIGETE GTFIVDSVELLMEE
<b>Length</b>	1155
<b>Number of 80 mers</b>	1076
<b>Number of Sequences with hits</b>	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---G-
Sequence 2 EDVPQPPVVSQFYIQGQVYCDTCRARFITEELSEFIPGAGVRLQCKD---GE

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

Sequence 1 -----
-----
Sequence 2 WVKPSLK FVLNTVNGTTRTINPLGFLKKEVLPKCPQVFNKLGMYPPNM

```

### iii. Allermatch database Sequence identity search of Cry1Ab protein

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

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 [Blot 1.0201](#), Sequence: [AAQ24541](#)

```

Sequence 1 -----
Sequence 2 MKFLLVAALCALVAIGSCKPTREEIKTFEQFKKVFVKVYRNAAEEEEARREH

Sequence 1 -----
Sequence 2 HFKEQLKWVEEHNGIDGVEYAINEYSMDSEQEFSEFHLGGGLNFTYMKME

Sequence 1 -----
Sequence 2 AAKEPLINTYGSLLPQNFEDWRQKARLTRIRQQGSCGSCWAFAAAGVAESLY

Sequence 1 -----
Sequence 2 SIQKQQSIELSEQELVDCTYNRYDSSYQCNGCGSGYSTEAFFKYMIRRTGLV

Sequence 1 -----
Sequence 2 EEENYPYNMRTQWCNPDVEGQRYHVSGYQQLRYQSSDEDVMTIQQHGPV

Sequence 1 -----YTDHAV-----
Sequence 2 VIYMHGSNNYFRNLGNGVLRGVAYNDAYTDHAVILVWGWTVQGVYWIIR

Sequence 1 -----
Sequence 2 NSWGTGWGNGGYGYVERGHNSLGINNFVTYATL

```

Sequence 1: Cry1Ab

Sequence 2: Allergen [Pha a 1](#), Sequence: [Q41260](#)

```

Sequence 1 -----GSAQGI-----
Sequence 2 MMKMVCS SSS SLLVVAALLAVFVGS AQGI AKV PPGPNITAEY GDKWLDA

Sequence 1 -----
Sequence 2 KSTWYGKPTGAGPKDNGGACGYKDVDKAPFNGMTGCGNTPIFKDGRGCGS

Sequence 1 -----
Sequence 2 CFELKCSKPESCSGEPITVHITDDNEEPIAPYHFDLSGHAFGSMARKGEE

Sequence 1 -----
Sequence 2 ENVRGAGELELQFRRVKCKYPDGTPKPTFHVEKGSNPNYLALLVKYVDGDG

Sequence 1 -----
Sequence 2 DVVAVDIKEKGDKDWIELKESWGAIWRIDTDPKLTGPFTVRYTTEGGTKA

Sequence 1 -----
Sequence 2 EFEDVIPEGWKADTHDASK

```

**Alignment 2**

Sequence 1: Cry1Ab

Sequence 2: Allergen [Salk 1.0302](#), Sequence: [AAX11261](#)

```

Sequence 1 -----
Sequence 2 QPIPPNPAELESWFQGAVKPVSEQKGLEPSVVQAESGGVETIEVRQDGSG

Sequence 1 -----
Sequence 2 KFRTISDAVKHVKVGNTKRVIIITIGPGEYREKVKIEGLHPYITLYGIDPK

Sequence 1 -----
Sequence 2 NRPTITFAGTAAEFGTVDSATLIVESDYFVGANLIVSNSAPRPAGKRKGA

Sequence 1 -----
Sequence 2 QASALRISGDRAAFYNCKFTGFQDTCDDKGNHLFKDCYIEGTVDLIFGE

Sequence 1 -----
Sequence 2 ARSLYLNTELHVVPGDPMAMITAHARKNADGVGGYSFVHCKVTGTGGTAL

Sequence 1 -----
Sequence 2 LGRAWFEEARVVFSYCNLSDAVKPEGWSDNNKPAAQKTIFFGYKNTGPG

Sequence 1 -AADKRV-----
Sequence 2 AAADKRVFYTKQLTEADAKTFTSLEYIEAAKWLPPPKV

```

**Appendix 10: Cry1Ab Gene Sequence**

5'-GTCCTTGGTCCGGTAGAGATAGGGACGGATTATAAGATCCAATGTTATCTGAGTC  
AAATATTTCCAATCAACTGCGCGCATCCGAGAAAAACAGTGAAAAACAAATGTATC  
GGGGTTAACCGTTGAGTAACGAAGCCAATTTAGTTACTACTCCGTTTGTCCGCCTTCT  
GGACCGCCACTTAGGCCGT-3'