PCR SCREENING OF GENETICALLY MODIFIED MAIZE IN BISCUIT PRODUCTS USING TNOS GENE

SABRINA GEORGE

SCHOOL OF HEALTH SCIENCES UNIVERSITI SAINS MALAYSIA

2022

PCR SCREENING OF GENETICALLY MODIFIED MAIZE IN BISCUIT PRODUCTS USING TNOS GENE

by

SABRINA GEORGE

Thesis submitted in partial fulfilment of the requirements for the degree of Master of Sciences (Forensic Science) Mixed Mode

SEPTEMBER 2022

ACKNOWLEDGEMENT

I would like to express my gratitude to my thesis supervisor, Dr. Nur Haslindawaty Binti Abd Rashid, who has helped, guided, and encouraged me a lot throughout the whole process in order to complete this study. She made many wise and valuable suggestions and recommendations, and I thank her for her efforts.

I am also grateful to my family for the supporting me throughout the years, giving me hope, motivation and love during my struggle times. I would like to thank my beloved friends too who always give me endless support and encouragement in this journey.

I would also appreciate those who involved directly or indirectly in the process of completing this study.

This project would not have been possible without the kind support and encouragement from everyone.

Thank you.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

bp	Base pair
CaMV	Cauliflower Mosaic Virus
CPB	Cartagena Protocol on Biosafety
CRM	Certified reference material
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium bromide
GM	Genetically modified
GMAC	Genetic Modification Advisory Committee
GMFs	Genetically modified foods
GMO(s)	Genetically modified organism(s)
HCl	Hydrochloric Acid
ISAAA	International Service for the Acquisition of Agri-biotech Applications
JKM	Department of Chemistry
Kbp	Kilo base pair
LMO	Living Modified Organism
$MgCl_2$	Magnesium chloride
Na_2EDTA	Sodium Ethylenediaminetetraacetic acid
NaOH	Sodium hydroxide
$NH_4(SO_4)_2$	Ammonium sulphate
NBB	National Biosafety Board
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism

TBE	Tris-Borate Ethylenediaminetetraacetic acid
TE	Tris-EDTA
USA	United State of America
UV	Ultraviolet
A260	Absorbance at wavelength 260 nm
A280	Absorbance at wavelength 280 nm
A260/A230	Absorbance ratio of wavelength at 260 nm to 230 nm
A260/A280	Absorbance ratio of wavelength at 260 nm to 280 nm
G	Grams
Min	Minutes
mL	Millilitre
mg/ µL	Milligrams per microliter
ng/ µL	Nanograms per microliter
rpm	Revolution per minute
sec	Seconds
µL	Microliter
%	Percent
°C	Degree Celsius
∞	Infinity

SARINGAN PCR BAGI JAGUNG YANG DIUBAHSUAI SECARA GENETIK DALAM PRODUK BISKUT MENGGUNAKAN TNOS GENE

ABSTRAK

Pada masa kini, tumbuhan yang diubah suai secara genetik (GM) digunakan secara meluas dalam pelbagai barangan komersial. Penggunaan kaedah kejuruteraan genetik yang canggih dalam pertanian telah membawa kepada peningkatan dalam pengeluaran tanaman yang diubah suai secara genetik serta produk makanan. Walau bagaimanapun, penggunaan teknologi genetik molekul kompleks untuk penciptaannya melalui adab nanobioteknologi untuk mendapatkan organisma yang diubah suai secara genetik, masih belum sempurna, yang menyebabkan beberapa risiko yang timbul daripada penciptaan organisma tersebut. Jagung adalah antara tumbuhan yang biasa diubah suai secara genetik dan telah diintegrasikan ke dalam rantaian bekalan makanan dan makanan. Dalam kajian ini, gen TNOS yang diubah suai secara genetik digunakan untuk menyaring sampel biskut yang dibeli dari pasaran sekitar Kota Bharu, Kelantan. Kit Makanan DNeasy Mericon telah digunakan untuk mengekstrak sampel biskut, jagung segar dan kawalan positif jagung GM. Walaupun bacaan kuantifikasi untuk hampir semua sampel menunjukkan kepekatan DNA yang boleh diterima dan nilai yang baik untuk ketulenan pertama. Bagaimanapun, tindak balas berantai polimerase (PCR) menggunakan gen *zein* dan TNOS menunjukkan semua sampel biskut tiada gen *zein* dan TNOS. Ini mungkin disebabkan oleh beberapa kemungkinan seperti kepekatan DNA yang sangat rendah, DNA yang diekstrak mungkin terdegradasi, gen berbeza yang digunakan untuk jagung GM, kepekaan PCR konvensional dan sensitiviti gel agaros. PCR positif untuk kedua-dua gen dilihat dalam kawalan positif jagung GM.

PCR SCREENING OF GENETICALLY MODIFIED MAIZE IN BISCUIT PRODUCTS USING TNOS GENE

ABSTRACT

Recently, genetically modified (GM) plants are employed extensively in a variety of commercial goods. The use of advanced methods of genetic engineering in agriculture has led to an increase in the production of genetically modified crops as well as food products. However, the use of complex molecular genetics technologies for their creation through nanobiotechnology manners to obtain genetically modified organisms, are not yet perfect, which causes a number of risks arising from the creation of such organisms. Maize is among the common genetically modified plants and has been integrated into the food and feed supply chains. In this study, the genetically modified TNOS gene was used to screen the biscuit samples bought from the market around Kota Bharu, Kelantan. DNeasy Mericon Food Kit was used to extract the biscuit samples, fresh maize, and GM maize positive control. PCR amplification was performed using *zein* and TNOS genes and results show all biscuit samples were absent of *zein* and TNOS genes. This could be caused by several reasons such as very low DNA concentration, the extracted DNA might be degraded, a different gene used for GM maize, the efficiency of conventional PCR and the sensitivity of agarose gel. Although the quantification reading for almost all samples showed an acceptable DNA concentration and good value for the first purity. Positive amplification for both genes was seen in the GM maize positive control.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Maize (*Zea mays L.*) is a vital component of animal feed and one of the most significant agricultural products. It is widely employed in industrial products across the globe, including the usage of maize for the production of renewable fuel (Shiferaw *et al*., 2011). Maize is the second most crucial carbohydrate source commodity after rice, which is very critical for food security (Halid, 2019). It is the foundation of the human diet in Latin American countries, particularly Mexico, where it is regarded as a main crop for the country's food supply (Santillán-fernández *et al*., 2021). Currently, maize is the world second most widely grown biotech crop. The ongoing development of biotechnology has allows the production of genetically modified organism (GMO) maize varieties with resistance to insects and herbicides, thus allowing for the production to rise by area (Saxena and Stotzky, 2001).

GMOs are living organisms where the genome has been altered by the insertion of an exogenous gene that is capable of expressing an additional protein conferring new characteristics or allowing the development of desired biological products, such as nutritional enhancement, herbicide resistance, or insect protection (Manzanares-Palenzuela *et al*., 2015). Selecting and removing genes of interest from other species, such as bacteria, then introducing the relevant DNA segment into the plant genome allows for these genetic alterations (Manzanares-Palenzuela *et al.*, 2015).

The commercial usage of GMO is on the rise in both industrialised and developing nations. Genetically modified (GM) agricultural technology has been widely employed in a variety of nations for over 20 years, with the four crops of canola, maize, cotton, and soybean being the most common (Brookes and Barfoot, 2018). In 2018, a total of 17 million farmers in 26 nations grew 191.7 million hectares of GM crops, which is an increase from the 189.8 million hectares that were planted in 2017. Of these 26 countries, 21 were developing countries and 5 were industrial countries (Wana *et al.*, 2021). The most important GM crop was soybean, which accounts for fifty percent of the total area of GM crops grown around the world. This is followed by maize (30.7%), cotton (13%), and canola (5.3%) (Wana *et al*., 2021).

The government of Malaysia acknowledges the importance of biotechnology in improving human health, particularly with regard to addressing the nation's needs in the areas of food production, agriculture, and medical care. In principle, the use of GM technology is permitted in Malaysia; however, this practise must be supported by precautionary and safety measures that are in line with Malaysia's international duties as well as its own domestic regulatory frameworks (Andrew *et al.*, 2018). In Malaysia, the study on GM is still in its infancy at this point., and most of the GM crops that have been licenced for release are only for food, feed, or processing (Andrew *et al*., 2018).

GMOs are becoming more widely used as food and in food items. However, some biosafety issues regarding the rapid transformation of GMOs are increasing throughout the advancement of genetic engineering, breeding sciences and biotechnology in the food industry (Gupta and Singh, 2017). The presence of GMOs sparks debates regarding its environmental risk, ethical concern, and food safety. As a result, it is important to determine if a product is GM or includes any GM ingredients to comply with the legislation's criteria. Although several industrialised nations have adopted legislation

requiring the labelling of items containing GMOs, the majority of food products on the market today must be tested for GMOs (Kamle and Ali, 2013). Labelling of GM crops and the goods made from them is required if the amount of GMOs in the food they contain is more than the permitted limit (Kamle and Ali, 2013).

The use of analytical technologies for the detection of GMOs is required to ensure that labelling requirements are satisfied. For this reason, countries all over the world require the development of technologies that are both functional and trustworthy for the identification of these organism. Generally, two methods are used to detect the GMO in food products, which are the deoxyribonucleic acid (DNA) based method which mainly works to detect the foreign DNA, and protein-based method which works to detect the cellular structure.

1.2 Problem Statement

Recent developments in GM crops have given rise to significant concerns over their efficacy and safety of the food. Human health and pest resistance issues have affected the GM seed sector, severely undermining its numerous favourable impacts. Furthermore, seed firms' poor science communication, a major absence of safety research, and contemporary fear of GMOs have further added to the difficulties (Raman, 2018).

The use of advanced methods of genetic engineering in agriculture has led to an increase in the production of GM crops as well as food products. However, the use of complex molecular genetics technologies for their creation through nanobiotechnology manners to obtain GMOs, are not yet perfect, which causes a number of risks arising from the creation of such organisms (Chaghakaboodi *et al*., 2021; Mahdewi *et al*., 2020).

GM crops are considered an “imperfect technology” with severe health concerns such as toxicity, allergenicity and genetic hazards associated with them (Raman, 2018).

1.3 Significant of Study

The growing number of cases of food fraud that have been reported all over the world has increased people's awareness of the necessity of identifying and verifying the components of food. For example, consumers' trust in products has been put in jeopardy due to the mislabelling of food items. In conjunction to this, it is crucial to screen for GMOs in order to fulfil the requirements of the relevant legislation and to address the public's perceived health concerns over GMOs *(Salihah et al*., 2016). As a result of the widespread dissemination of GM crops around the world (James, 2009), there has been an increase in the practise of falsifying food labels and counterfeiting products that contain GMO ingredients. The growing possibility that unintended contamination of non-GMO food with GMO residues may occur during the processing and manufacture of food, resulting in a decrease in the market value of the items (Ruther, 2009). Because of this, effective detection of GMOs is very necessary in order to protect the authenticity of food items.

In biscuit production, corn is one of the ingredients used in the form of flour. This study is intended to screen the presence of GM maize in biscuit products that are sold around the Kota Bharu market. The result obtained can be used to increase the customer’s confidence level against food products especially biscuits because it is one of the most frequent products being bought by the consumers.

1.4 Research Objective

1.4.1 General Objective

This study is aimed to identify the presence of genetically modified maize in biscuit products.

1.4.2 Specific Objective

1. To compare the DNA extraction method used for biscuit products.
2. To amplify the extracted gDNA using *zein* and TNOS primers.
3. To assess GM maize through agarose gel electrophoresis of amplified product.

CHAPTER 2

LITERATURE REVIEW

2.1 Maize (*Zea mays L.*)

Commonly known as corn, maize (*Zea mays L.*) is one of the most important cereal crops grown worldwide. Most of the world's population relies on maize for sustenance and becoming a staple crop in many parts of Africa and recently gained popularity as a culinary staple in parts of Asia, including China, India, and Indonesia. However, maize is not simply a human food resource but also one of the important ingredients used for animal feed (Gwirtz and Garcia-casal, 2014). In addition, maize is a common raw material, additive, and ingredient in the food and feed industries (Kommers *et al*., 2016). As a component of the human diet, corn can be consumed fresh as corn ears, used as the main ingredient in processed foods (polenta, tortillas, burritos), or as a snack food (popcorn and corn chips) (Kommers *et al*., 2016). There are several packaged goods available in the market that include corn including cereals, sweets, jams, syrups, sauces, snack foods, canned fruits, prepared meats, and drinks.

Although maize allergy is not considered a common dietary allergen, it is increasingly being explored by physicians throughout the world (Bernstein *et al.*, 2003; Stevens *et al*., 2011). The maize allergy is treated by rigorous avoidance of maize components, and it is essential to understand the presence of allergens in meals to avoid health complications. As maize is one of the top four GM crops, the exposure of consumers to maize allergy is reported to increase (Onishi *et al*., 2005; James, 2009). Therefore, accurate identification of maize is necessary for the labelling and monitoring of GM foods in order to satisfy customer demand for choice.

Anklam *et al*., 2002 have stated there are different molecular approaches for identifying maize that have been developed for many years. The DNA-based polymerase chain reaction, often known as PCR, is widely regarded as the most efficient method for analysing foodstuffs. The features of DNA as the molecule are more stable compared to protein, thus making it become a more preferred genetic marker to be used for the identification of processed food products.

2.2 Industrial Maize Processes

Industrial processing of maize into food items may be broken down into two broad groups namely dry milling and wet milling (Ekpa *et al*., 2019). The components such as starch, protein, oil, and fibre are extracted from maize in their purest forms during the wet milling process (Gwirtz and Garcia-casal, 2014; Kumar *et al*., 2022). Wet maize milling often results in goods and co-products that are not utilised directly by the customer and frequently require additional industrial processing before consumption. Small-scale commercial or domestic production of wet maize milled goods is uncommon. Starch is the basic product that may be refined into a wide range of sweeteners, both liquid and dry, and then marketed in several formats (Gwirtz and Garcia-casal, 2014; Kumar *et al*., 2022). The process of wet milling is illustrated in Figure 2.1.

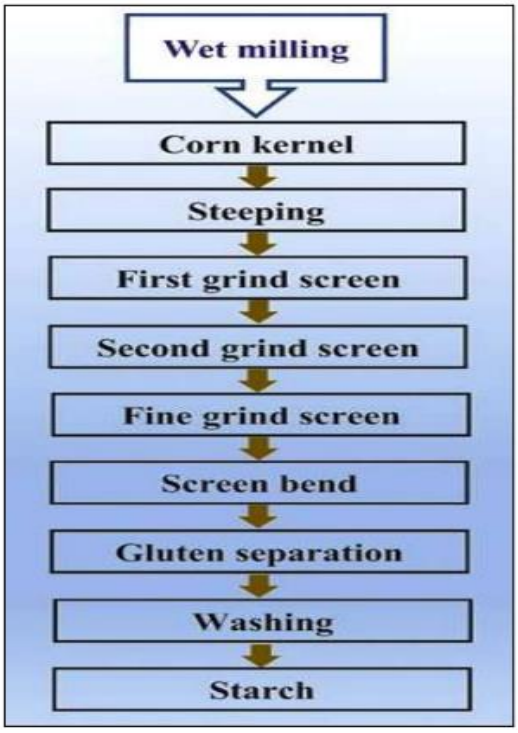

Figure 2.1: The wet milling processes (Zhang *et al*., 2021).

Industrial dry maize processing involves the use of nixtamalization which is referring to alkaline processing that involves boiling entire ears of maize in a pot filled with water and treated with calcium oxide (Ekpa *et al*., 2019). Maize can be processed in a variety of ways, including grinding the whole kernel, grinding fractions of the kernel, or adding additional maize components (Gwirtz and Garcia-casal, 2014; Kumar *et al*., 2022). In contrast to the dry processing of wheat flour, the wet processing of alkali-treated maize necessitates specific equipment for the handling of moisture, chemicals, and heat. Raw maize and the dry end-product are processed using the same equipment used for other dry bulk materials. The resulting intermediate product might be dried and sold as a commercially processed food item for consumers (Gwirtz and Garcia-casal, 2014; Ekpa *et al*., 2019). The process of dry milling is shown in Figure 2.2.

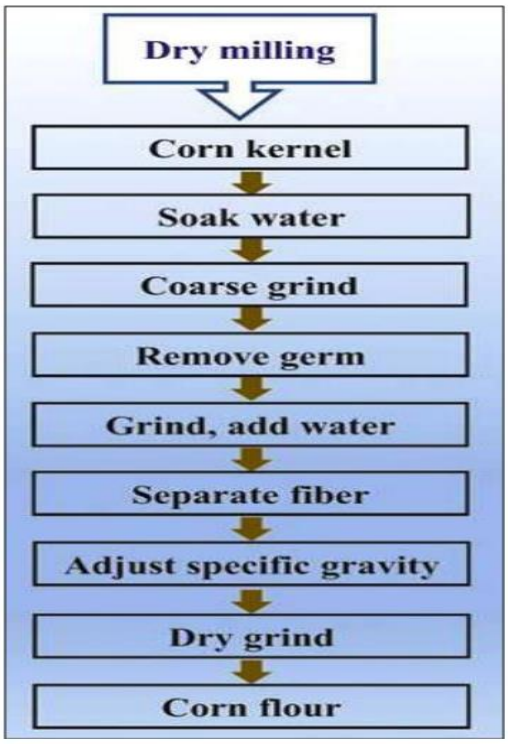

Figure 2.2: The dry milling processes (Zhang *et al*., 2021).

2.3 Genetic Modified Organism (GMOs)

Controversies around GMOs have raged for decades. There are reports that the regulatory system for GMOs in some nations is rather strict. GM crops are a by-product of precision breeding techniques, a subset of advanced breeding technology (Eriksson *et al.*, 2018).

Multiple self-crossings (inbreeding) are used to stabilise favourable features in traditional breeding, which began with the straightforward mating of high-performing organisms. Later, mutation breeding (modification of the genetic makeup of plants) was developed to provide variance in population performance. Since the 1960s, ethyl methanesulfonate (EMS), an alkylating chemical was used and it reacted with cell components and produce genetic alterations in organisms (Krieg, 1963). In the middle of the 20th century, ionising electromagnetic irradiations (X- and gamma-rays) were also

utilised to induce random mutations in the genes of plants from which elite lines with desirable characteristics were selected for subsequent breeding (Ulukapi and Nasircilar, 2015).

The expansion of plant genetics and the discovery of the transferability of DNA and RNA in the 1970s (Chassy, 2007) led to the creation of biotechnology using the "genetic engineering" approach. Selecting elite lines and engaging in several generations of inbreeding led to these subsequent advancements, not random mutations of DNA. GMOs containing DNA from unrelated species have become a reality because of this discovery. As a result, precision genetic engineering (GE) was developed, and precise site targeted modifications were made (Nakayama *et al*., 2014).

While GMOs were first applied to bacteria, the greatest economic utility has undoubtedly been in the food industry. In 1980, genetics engineer at General Electric was awarded the first GMO patent for a microbe that could be used to clean up oil spills since it consumes crude oil. The first GMO medicine, Humulin, a version of Insulin developed in a laboratory, was authorised by the US Food and Drug Administration (FDA) in 1982 (Woolsey, 2012). Flavr Savr tomatoes is created by Calgene to have longer storage life and were the first commercially marketed food product after receiving FDA approval in 1994. Although this specific GMO food product was not well accepted and was discontinued in 1997 (Winerip, 2013), it was quickly followed by numerous others (such as soy and maize) that are now commercially accessible throughout the USA today (Fernandez-Cornejo and Wechsler, 2014).

2.4 Transgenic Plant

Transgenic plants or often known as GM plants are plants whose genomes have been modified using techniques called genetic engineering. This modification might take the form of the introduction of a foreign gene or the removal of a particular damaging gene (Rani and Usha, 2013). It is possible that a foreign gene introduced into a plant came from a completely other species, or perhaps an entirely different kingdom. As a result of the rapid advancement in plant molecular biology and genetic engineering technologies, transgenic plants have essential agronomic characteristics such as resistance to pests and drought tolerance. The creation of crops with ideal characteristics has improved the quality and increased production where it becomes the main goal of transgenic plant research.

Typically, GM plants can be created based three basic components: a transgene, a promoter, and a terminator. Although numerous possible promoter and terminator elements have been found, the CaMV 35S promoter which is derived from the 35S RNA of the phytopathogenic cauliflower mosaic virus, is the most often employed (El-Kindiy and Mpoloka, 2017). The 35S promoter (P35S) from the Cauliflower mosaic virus (CaMV) and/or the NOS terminator (TNOS) generated from the nopaline synthase from the soil-borne bacteria *Agrobacterium tumefaciens* are both potential candidates for GM plants. Ti plasmids are utilised in roughly 80% of all genetic constructs for commercial purposes in GM plants (Bak and Emerson, 2020).

For example, the genetic modification of a commercially available soybean involves the introduction of a glyphosate-resistant 5-enol-pyruvyl-shikimate-s phosphate synthase (EPSPS) gene, with the cauliflower mosaic virus-derived 35S-promoter and the nopaline synthase (NOS) transcription terminator sequence used for EPSPS gene expression. This allows the soybean to be resistant to the herbicide glyphosate. (Conner

et al., 2003; Sithole-Niang *et al*., 2004). Both 35S and NOS sequences are the most prevalent gene from promoter and terminator, respectively used in commercially cultivated transgenic crops. As these genetic components do not exist naturally in agricultural crop sources, the ability to detect them will allow for the detection of the great majority of GMO plant material (El-Kindiy and Mpoloka, 2017).

2.5 GMOs in Malaysia

To date, plant biotechnology has not been well developed or manufactured in Malaysia. However, the number of GE events that have been allowed for commercial usage in the country is in growing trends. According to ISAAA, (2022), a total of 44 GE events were commercially available for import and sale in Malaysia till August 2022 as compared to the 38 events of GE in 2018, which showed an increment of around 14% (Verzani *et al*., 2018). Since this study focused on the GM maize screening, different type of approved GM maize exists in the Malaysian market as shown in Appendix A.

The issue of GE labelling has been raised in 2013 by the Malaysian Ministry of Health regarding the rules for GE, however, no action has been taken and implemented (Verzani *et al*., 2018). The rules or guidelines for GE are important to be declared to protect the public from potentially harmful foods and to provide the food business and consumers with correct information. As stated by Verzani *et al.* (2018), any items with more than three per cent of GE material must be labelled as such as per the recommendations, but this rule has not been implemented and is only kept as a record.

In 2017, approximately two-thirds of Malaysia's maize came from Argentina and Brazil, while the 50% of 800,000 tonnes of soybean was imported from the United States (Verzani *et al*., 2018). The poultry business in Malaysia relies on imported maize due to the inadequate local supply of feeds. However, most of the soybeans imported are used

to make food items like soymilk and tofu which accounted for 15% of the total and the remaining was served as fodder for farm animals (Ali *et al*., 2020). Three world leading producers of maize and soybeans are the United States, Brazil, and Argentina with 80% of the total amount of production being genetically modified (Wana *et al*., 2021).

The term "low level presence" or LLP has been developed to characterise the inadvertent presence of modest quantities of unapproved GM crops. When remnants of these unapproved GM crop occurrences are identified in grain shipments, ingredients, or final food products, trade disruption may result (Kalaitzandonakes *et al*., 2014). It can lead to hefty fines, lost income on the entire grain cargo, costly testing and clean-up, unsold or damaged grain or seed, product recalls in importing nations, and the loss of export market share when the importing country sources grain from another country (Kalaitzandonakes *et al*., 2014).

2.6 Health Risks Associated with GM Food Consumption

Numerous pieces of evidence from the scientific community showed that the animals that were fed GM crops suffered from health problems or even perished. Rats that were given transgenic potatoes or soya had abnormal immature sperm whereas animals such as cows, goats, buffalo, pigs, and other livestock that were fed Bt-maize, GM cottonseed, and specific biotech corn exhibited issues such as preterm births, abortions, infertility, and many fatalities; and rats that were given transgenic potatoes or soya had abnormal immature sperm (Fares and El-Sayed, 1998; Ewen and Pusztai, 1999; Hashimoto *et al*., 1999; Momma *et al*., 1999; Velimirov *et al*., 2008). However, these issues have been disputed because the involved company in the biotech crops has found no evidence of any harmful consequences caused by GM crops based (Munro, 1999).

During the last ten years, the prevalence of foodborne diseases like soya allergy has increased in both the United States and the United Kingdom (Daniel and Berger, 2004). Additionally, the United States is experiencing an epidemic of Morgellons disease (Ho and Cummins, 2008). In India, there have also been reports of hundreds of villagers and cotton workers showing skin allergies (I. L. Bernstein *et al*., 1999; Kurunganti, 2008). Recent studies have demonstrated that *Bacillus thuringiensis* (Bt) corn expresses an allergenic protein that can alter the immunological responses of the body as a whole (Vazquez-Padron *et al*., 2000; Pasini *et al.*, 2002;).

It has been stated that transgenic crops pose a possible danger to human health (Selgrade *et al*., 2003; Devos *et al*., 2005;). Mutations that arise throughout the course of genetic modification have the potential to alter bioactivity and cellular structure. Cellular changes brought on by these sorts of alterations might have a deleterious effect on human health. Tissue cultivation protocols and the existing methodologies utilised by genetic engineers are viewed as harmful and potentially cause mutagenic. Transgenic products might undergo unexpected changes to their DNA, proteins, and metabolic processes as a result (McFadden and Lusk, 2016; Eriksson *et al*., 2018;). Toxicity, allergy development, the altered nutritional value of transgenic goods, nutrient decrease, facilitating the onset of chronic illness, and damage to bodily tissues and organs are all possible outcomes of the unpredictability of cellular level alterations. Several studies suggest that these items are not essentially recommended to be used because of their health impact on human (Selgrade *et al*., 2003; Devos *et al*., 2005; Delaney, 2015; Boccia *et al*., 2018).

2.7 *Zein* gene marker for Maize

Maize is the most significant cereal crop in the world, both as a source of food and animal feed since it is rich in endosperm storage proteins and starch. The zein protein that is found in maize endosperm is a member of the prolamin family. Prolamins are the most common form of protein that can be found in cereal seeds including wheat, maize, sorghum, rice, and barley (Holding, 2014). The functional research of maize and sorghum's prolamins are vital from both an economic and an environmental point of view because these two grains are the repertoires of essential amino acids and biological nitrogen. Zein accounts for around 70% of the entire amount of protein found in maize seeds, which totals 10% overall. In maize endosperm, zein proteins exist in protein bodies (PBs) inside endoplasmic reticulum (ER)(Khan *et al*., 2019).

Seed storage proteins primarily function as biological reserves of amino acids that can be mobilised and used for seedling growth. These amino acids can be used by the seedling. Albumins, globulins, glutamines, and prolamins are only few of the several types of storage proteins that have been identified in maize kernels. These proteins were separated into their respective categories based on their solubility in various solvents (Shewry *et al.*, 1995; Yongrui Wu and Messing, 2014). The prolamins found in maize grain are referred to as zeins. There is one major class of zeins, known as α-zeins, and three minor groups (β, γ and δ) (Sofi *et al*., 2009). These four types α, β, γ and δ constitute about 50-70% of maize endosperm and are essentially rich in glutamine, leucine, and proline, and poor in lysine and tryptophan (Sofi *et al*., 2009).

There is no other part of the plant than the seed that has ever been found to contain zeins. Zeins are the most prevalent type of protein found in maize endosperm, and their levels of expression are exceptionally high in this tissue. The existence of amplifiable maize DNA can be verified using plant-specific primers which are specifically designed

as confirmation for the intrinsic *zein* gene (Nguyen and Michael, 2009; Rabiei *et al.*, 2013).

2.8 Genes Used for Detection of GMO

According to Ahmed, (2002), *cauliflower mosaic* virus (CaMV) 35S promoter, nopaline synthase (nos) terminator, or the kanamycin-resistance marker gene are the genetic components that are present in the majority of the GMOs. The *cauliflower mosaic* virus or often known as CaMV, is a member of the family of circular, double-stranded DNA viruses known as *Caulimoviridae*. Most plants that are prone to infection are members of the Brassicaceae family such as radish, turnip, canola, mustard, cauliflower, broccoli, and cabbage. The 35S promoter (P35S) of the *cauliflower mosaic virus* (CaMV) is used widely for identifying GMOs. There are 24 distinct detection techniques that have been published so far, and each of them targeted a different area of the P35S promoter (Yuhua Wu *et al*., 2014). There is a larger percentage of P35S promoters present in transgenic crops that are relevant to commercial agriculture. Officially 67 out of 102 sanctioned commercial GM events has used the P35S promoter (Yuhua Wu *et al*., 2014). Many different GMO screening techniques have been developed and published as a result of the significance of the P35S promoter in the screening and detection of GMOs (L. W. Pan and Zhen, 2004; Cao and Xu, 2012).

2.9 Methods for GMOs Detection

There have been many different ideas put up for ways to identify GMOs, such as detecting altered DNAs, RNAs, proteins, metabolites, or phenotypes (C.-H. Lin and Pan, 2016). The selection of the method is depending on factors such as the analytical

parameters used and the cost that will be involved. Current methods are based on DNA and proteins (Alocilja, 2014).

The DNA-based technique involves either qualitative or quantitative analysis of the three categories of recently introduced genes. Immunoassays, such as ELISA, which is the most traditional assay explored and published by many organisations, are used to detect, and analyse crops at the trait level using protein-based assays. These assays are used to detect and analyse crops at the trait level (Lai *et al*., 2017; Y. Lin, Zhou, and Tang, 2017; Y. Lin, Zhou, Tang, *et al*., 2017). Protein-based approaches are typically regarded as inadequate for the detection of a wide variety of GMOs, particularly for processed foods. This is because protein-based methods have a lower stability (Holst-Jensen *et al*., 2016; Lian and Zeng, 2017). As a result, techniques based on polymerase chain reaction continue to be regarded as the industry's gold standard for GMO detection (C.-H. Lin and Pan, 2016).

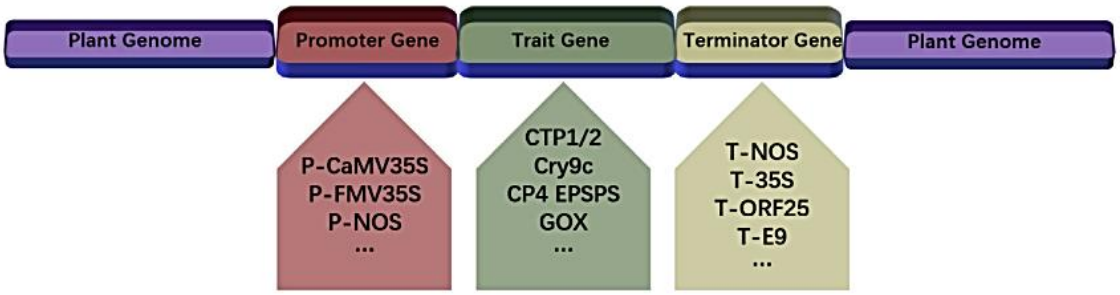

Figure 2.3: Schematic of GMOs gene cassettes, consisting of a promoter gene, a trait gene, and a terminator gene. Some examples are listed below each part.

2.9.1 DNA-based method

DNA is a stable molecule that can still be identified even after it has been fragmented, damaged, or denatured to some degree. This is in contrast to the behaviour of proteins. Even in highly processed food matrices, it is feasible to identify, extract, and do further analysis of DNA (Alocilja, 2014). The polymerase chain reaction (PCR) is now the most prominent DNA-based approach and the GMO detection method that is most widely accepted. Due to its high sensitivity, high specificity, and wide variety of gene constructions, PCR technology is the method of choice for many analytical laboratories that are interested in the detection of GMOs (Ahmed, 2002; Anklam *et al*., 2002; Giovannini and Concilio, 2002; Holst-Jensen *et al*., 2003).

PCR-based approaches have been implemented for the purpose of identifying and quantifying GMOs in food matrices, in accordance with ISO 24276:2006. PCR approaches, which target the stable integration site of foreign DNA components in a genome, make it possible to detect, identify, and quantify GMOs. This is possible due to the fact that such a combination does not occur naturally (Grohmann *et al*., 2019). This is partly attributable to its capacity to amplify certain DNA segments using highly processed materials as the starting point (Marmiroli *et al*., 2008). PCR-based GMO detection methods may be further subdivided into the areas of element-specific, construct-specific, and event-specific PCR (Holst-Jensen *et al*., 2012).

The element-specific PCR method is used to target a particular DNA sequence, such as a gene's promoter, terminator, coding region, or intron. This approach provides more accurate results. While by using a construct-specific PCR approach, it can detect the junction of two transgenic elements even though these elements do not occur in nature (Holst-Jensen *et al*., 2012). The approach of event-specific PCR, also known as GMO-specific event PCR is focusing on the detection of one of a kind chimeric sequence that

are produced whenever a transgenic construct is successfully incorporated into a host genome. The event-specific PCR approach, such as real-time PCR (qPCR), is particularly appropriate for detecting and quantifying GMOs. Additionally, this method has been widely utilised for regular GMO detection in law enforcement (Holst-Jensen *et al*., 2012).

Throughout most instances, the process of analysing GMOs using PCR begins with a qualitative screening to determine the presence of genetic components that are often associated with GMOs. These genetic elements include the *cauliflower mosaic* virus 35S promoter (p35S) and the nopaline synthase terminator (tNOS) (Holst-Jensen, 2009; Holst-Jensen *et al*., 2012). The usage of quantitative detection techniques such as quantitative competitive PCR (QC-PCR), real-time PCR, and ELISA systems have assist the identification of GMO from the food product.

2.9.2 Protein-based Method

In most cases, the synthesis of certain proteins is what differentiates GMOs from non-GM organisms. For protein-based analysis, the needed target is intact proteins; in the case of GMOs, this can be accomplished through the use of immunoassays (Alocilja, 2014). The protein-based method is an immunoassay technique that is based on antibodies. This method is a tested methodology for qualitative and quantitative detection of protein of a known target analyte (Brett *et al*., 1999). Antibodies are proteins that selectively attach to the material that prompted the creation of antibodies in animals. Antibodies are formed in the serum of animals in reaction to foreign compounds known as antigens (Alocilja, 2014). Depending on the specificity of the detection technique, either monoclonal (very specific) or polyclonal (usually more sensitive) antibodies might be utilised. The limit of detection (LOD) of a protein immunoassay can predict the

presence of recombinant protein in up to 1% of GMOs based on typical concentrations of transgenic material in plant tissues (>10 g per tissue) (Stave, 2002).

The enzyme-linked immunosorbent assay (ELISA) and the use of lateral flow devices (LFD) are the two protein-based techniques that are utilised the most frequently for the detection and quantification of GMOs (Stave, 2002). Protein-based methods have fewer applications than DNA-based methods, but they offer a number of benefits that cannot be ignored. For example, LFDs are not only affordable but also easy to use and capable of providing a speedy detection of the presence or absence of proteins that are coded by inserted GMO genes. On-site usage (for example, in grain elevators) is possible with only the barest necessities in terms of knowledge and apparatus (Jang *et al*., 2011). LFDs can also be purchased in comb form for the purpose of detecting numerous GMOs simultaneously. They are particularly helpful for screening plant seedlings for the presence of certain GMO features, and they may also be used for examination of grain samples in which the proteins have not been sheared. Both of these applications take place during the seedling stage of plant growth (Jang *et al*., 2011).

ELISA is used to address the quantitative aspect of protein-based approaches. This assay gives quantitative test findings by employing established reference standards and optical plate readers (Lipton *et al*., 2000). The great specificity of the resulting immunological reaction is the primary benefit of ELISA. This specificity makes it possible for antigenic substances to be precisely recognised, even in the presence of chemicals that may interfere with the test. In addition to this, it provides a high level of automation as well as sample throughput (Alocilja, 2014). However, while these tests cannot be used to reliably analyse processed materials, they are well suited to the inspection of raw materials and, as a result, might be useful in the identification of live transgenic animals. The ELISA and protein strip tests are the methods of choice for

distinguishing between GM and non-GM entities, as well as for identifying the modification event. This is due to the fact that these tests have quick turnaround times and only require a relatively small investment in both equipment and personnel (Alocilja, 2014).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemical and Reagents

All the chemicals, reagents and consumables used in this study are shown in Appendix B.

3.1.2 Instruments and Apparatus

All the instruments and apparatus used in this study are shown in Appendix C.

3.1.3 Reagents Preparations

3.1.3.1 10% Sodium Dodecyl Sulphate (SDS)

A total of 10 g of SDS powder was dissolved in 90 mL of sterile deionised distilled water. This was done on the hot plate to assist dissolution. The pH of the solution was then adjusted to 7.2 by adding 1 M hydrochloric acid (HCl) solution. The final volume of solution was adjusted to 100 mL by adding sterile deionised distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.2 1 M Tris-Hydrochloride (Tris-HCl) pH 8.0

A total of 121.14 g of Tris-base was dissolved in 800 mL of deionised distilled water. The pH was adjusted to 8.0 by adding 1 M HC1. The final volume of solution was

adjusted to 1000 mL by adding sterile deionised distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.3 0.5 M Ethylenediaminetetraacetic acid (EDTA) pH 8.0

A total of 186.1 g of EDTA, disodium salt, dihydrate (Na_2EDTA) powder was dissolved in 800 mL of deionised distilled water on a hot plate to assist dissolution. The pH of the solution was adjusted to 8.0 by adding 1 M sodium hydroxide (NaOH) pellet of around 20 g. The final volume was adjusted to 1000 mL. The solution was then autoclaved and stored at room temperature.

3.1.3.4 5 M Sodium Chloride (NaCl) Solution

A total of 58.44 g of sodium chloride powder was dissolved in 200 mL of deionised distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.5 Chloroform: isoamyl Alcohol (24:1)

A total of 24 mL of pure chloroform was mixed with 1 mL of isoamyl alcohol in an amber bottle wrapped with aluminium foil. The mixture was stored at room temperature.

3.1.3.6 70% Ethanol

This solution was prepared by diluting 35 mL of absolute ethanol with 15 mL of sterile deionised distilled water and stored at room temperature.

3.1.3.7 Orange G Loading Dye

A total of 0.125 g of orange G powder was added to 15 g of glycerol solution and the solution was adjusted to 50 mL by adding sterile deionised distilled water. The solution was aliquoted into 1.5 mL microcentrifuge tube and stored at -20°C.

3.1.3.8 2% Hexadecyltrimethylammonium bromide (CTAB) Solution with pH 7.2

A total of 2 g of CTAB powder was dissolved in 90 mL of deionised distilled water. This was done on the hot plate to assist dissolution. The pH of the solution was then adjusted to 7.2 by adding 1 M HCl. The final volume of solution was adjusted to 100 mL by adding sterile deionised distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.9 2% Hexadecyltrimethylammonium bromide (CTAB) Solution with pH 8.0

Two hundred millilitres of the solutions were prepared by dissolving 16.37 g of NaCl, 4.000 g of CTAB, 2.4230 g of Tris, 1.4899 g of Na_2EDTA and 2.000 g of polyvinylpyrrolidone (PVP-40) in 150 mL of deionised water. The pH was adjusted to 8.0 with addition of concentrated HCl or NaOH. The final volume was adjusted to 200 mL with deionised distilled water. The solution was then sterilised using autoclave.

3.1.3.10 Proteinase K (20mg/mL)

A total of 20 mg of proteinase K powder was dissolved in 1 mL of sterile deionized distilled water. The solution was then stored at -20°C.