COMPARATIVE ANALYSIS OF GENETICALLY MODIFIED MAIZE BY IMPLEMENTATION OF A HALF – SEED EXTRACTION TECHNIQUE

BY

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Student number: 195 00 273

A dissertation submitted in partial compliance with the requirements for the Masters of Technology: Biotechnology degree in the Department of Biotechnology and Food Technology, Faculty of Engineering, Science and the Built Environment, Durban University of Technology.

Supervisors : Dr. Kugen Permaul

: Mrs. T. Erasmus

*** SUBMISSION APPROVED FOR EXAMINATION**

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DR K PERMAUL (Ph. D) **DATE**

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ABSTRACT

Maize is one of South Africa's major agronomic crops due to its use as a staple food. Stalk borer has been a major pest and is responsible for losses in the area of maize production. The introduction of genetically modified maize has provided a solution to this problem. This study was undertaken to employ a half – seed technique that would allow one to complete screening at the seed level and also have the ability to plant the rest of the seed. The analyses to be undertaken also included various molecular analyses on the storage proteins and DNA extracted from the seed fraction.

Four maize lines (yellow and white) and two hybrids (white) were analysed using the half – seed extraction protocol and the extracted protein and DNA were analysed using ELISA, IEF and PCR. The Bt trait could not be detected using IEF. However, the IEF results provided an in-depth view into the genetic purity, as well as homogeneity of the samples.

The study resulted in the optimization of the half – seed technique. The ELISA analyses provided results in terms of presence/absence of the trait, as an inexpensive prescreen to large quantities of samples that required further screening in real time - PCR. The real - time PCR results allowed one to select plants that were homozygous. The data obtained

from this study resulted in a streamlined process for the screening of maize lines for genetic purity and for the presence of the Bt trait for the use in the production of new hybrids.

PREFACE

The experiments in this dissertation were done at the Biotechnology Laboratories at Pannar Research and have been under the direct supervision of Dr. Kugen Permaul and Mrs. Tertia Erasmus during the period of January 2002 to November 2006. The work presented in this dissertation has not been submitted to any other tertiary institute. The interpretation of results in this dissertation is those of the author and does not necessarily reflect those of the Durban University of Technology.

Fernando Pienaar

12 February 2007

DEDICATION

This work is dedicated to my parents who have made it possible for me to accomplish this achievement. Thank you for all your love, support and sacrifice.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Maize breeders are continuously seeking to improve the quality of existing varieties by incorporating traits such as drought tolerance, yielding ability, resistance to diseases etc. as they produce seed for commercial use. The development of molecular techniques have created opportunities for breeders to incorporate these technologies into traditional breeding to decrease the time it normally takes to produce a new variety. Biotechnology is continuously developing and new technologies are always being introduced to improve analysis. In order to be competitive, plant breeders are eager to change their breeding programmes with these developments. Phenotypic selection has been the traditional way of selecting plants as breeding material and now breeders are incorporating molecular techniques into their programmes.

1.1 **History of Maize**

The oldest maize remains were discovered in caves in the Mexican Tuacan Valley, indicating that maize as used as a crop 6000 years ago. When Columbus discovered the New World in 1492, maize was being cultivated by Amerindian tribes from southern Canada to southern Chilli, as well as in Haiti and Cuba. The maize originally planted by the native people of South Africa were flint types, as were those which were to be established in the early Cape Colony. The old varieties were obtained by mass selection, i.e. by retaining only the best ears obtained in a particular environment. All of these were open-pollinated (van Rensburg, 1994).

Maize cultivars and landraces are known to be diploid, i.e., they contain a set of 10 paired chromosomes, which contain the DNA or genetic material, and are interfertile to a large degree. However, some evidence for genetic incompatibility exists within the species (e.g., popcorn X dent crosses, etc). Some popcorn hybrids are dent-sterile and cannot be pollinated by ordinary types of dent or sweet corns (Rooney and Serna-Saldivar, 1987). *Zea mays* has been domesticated for its current use be selection of key agronomic characters, such as non-shattering rachis (ear), grain yield and resistance to pests (Kiesselbach, 1949).

Maize, commonly known as mealies in South Africa, originated in Mexico, which is the center of diversity for maize. Maize diversity is directly related to food security, with maize a staple food for many poor communities in southern Africa. In 2001, permission was granted for genetically engineered (GE) white maize to be grown in South Africa, the first country ever to introduce a GE staple food for direct human consumption. In 2004, GE maize accounted for an estimated 35% of all maize grown in South Africa. (Pschorn-Strauss *et. al*., 2004).

1.2 **Maize as a Food Source**

Maize plays a vital role in food security for many poor households and is a critical food and cash crop with a per capita consumption of over 100 kg. Both large and small-scale commercial farmers produce maize. Maize production is unstable because of erratic rainfall, and yields range from 1 to 4 tons/ha. Trends towards lower rainfall in the drier

areas of southern Africa suggest these areas are becoming increasingly unsuitable for maize production. In South Africa, the area planted with maize has decreased with the deregulation of the industry, from over 5 million ha in the mid to late 1980s to around 3.5 million ha in 2004 (Pschorn-Strauss *et al*., 2004). South Africa has about 8000 commercial maize farmers. Since deregulation of the industry, the price of maize has been derived from international prices and dependent on the exchange rate. The value of the maize crop varies from below 10% to over 20% of total agricultural production in the country. Large-scale maize production is highly capital intensive and due to rising input costs, farmers become increasingly tied to credit, input suppliers and marketing agents. White maize is preferred for human consumption and is also used for animal feed, with yellow maize used mainly for animal feed and for some processed foodstuffs, such as cereals. This is mainly due to preference and tradition (Schroder, 2006). Maize is also used to produce starches and syrups used in a wide array of foods and industrial products. African Products are a major processor of maize and purchases about 10% of the annual maize crop, contracting farmers to grow GM - free maize. South Africa exports and imports maize and maize products. Maize is also an important input for the poultry industry, which is South Africa's second largest agricultural sector (Pschorn-Strauss *et al*., 2004).

1.3 **Integration of Plant Breeding with Plant Molecular Biology**

Plant breeding involves the exploitation of genetic variation in plant chromosomes and its recombination into new permutations. To understand the nature and the origin of the

variation the plant breeder uses, it is essential to be able to look at the chromosomes and genes at the molecular level. The development of techniques in molecular biology and, in particular, the complete purification of large amounts of specific DNA sequences by cloning bacteria, has made this possible (Owens, 1983). Maize research, development and extension work is dominated by industry, with Pannar the leader in developing local varieties. The Grain Crops Institute of the projects on maize on request by industry. The ARC also responds to the needs of small-scale farmers to some extent, making available OPV varieties on request. The National Maize Producers' Organization (NAMPO) lobbies for farmers' interests, including research and development (Pschorn-Strauss *et. al*., 2004).

Breeding progress in maize continues to increase yield at a rate of 1 to 2% per year, with additional gains made for disease resistance, maturity, standability and production efficiency. Virtually all gains are due to utilization of polygenic factors not readily handled by currently available molecular procedures. Molecular genetics will not add to routine breeding practices until this is overcome (Goodman, 2003).

1.4 **Application of Molecular Biology to Speed up the Processes of Crop Improvement**

Plant breeding is based on the principles of Mendelian genetics. In the past, plant breeding was something of an art and selection of superior genotypes of a particular crop depended to a great extent on subjective decisions made by the breeder. With increasing

knowledge of the genes underlying useful traits, plant breeding has become a more directed and scientific activity. This is, in part, a result of the generation of molecular maps of crop genomes, extensive sequencing of expressed sequences and of genomic sequences and of study of genome organization, repetitive and non-coding sequences and ability to identify polymorphisms at particular loci which can be exploited as molecular markers if they are closely linked to a useful trait (Walker and Rapley, 2000).

The Monsanto Company has developed a genetically modified line of field corn/maize that produces a protein throughout the maize plant that is toxic to certain insect species. The resulting plant line is identified commercially as "MON810" and the crop is one of several transgenic maize varieties generally referred to as Bt-corn or Bt-maize. The molecule produced through the genetic alteration is identified as Δ-endotoxin or the Cry1Ab protein.

1.5 **Transformation of Maize**

MON810 was developed by co-transforming maize with two vectors, one carrying a synthetic *cry1Ab* gene and the second bearing the two herbicide resistance genes (EPSPS and *gox*). The genetic alteration that produced MON810 incorporated a truncated form of the synthetic form *cry1Ab* gene from the bacterium, *Bacillus thuringiensis* subsp. *kurtsaki*, into a Hi-II type maize line. The modified *cry1Ab* gene maintained its ability to produce the delta-endotoxin in plant tissues at levels that are toxic to certain lepidopteran insects. The *cry1Ab* gene is expressed from the 35S promoter (E35S) derived from

cauliflower mosaic virus, a known plant pest, and is joined to the nopaline synthase 3 transcription terminator, NOS 3', derived from *Agrobacterium tumefaciens*, a plant pathogenic bacterium. These genetic elements (promoter, gene, termination sequence) are all necessary for proper expression of the introduced trait (i.e., Bt protein) in the maize plant (AGBIOS, 2004).

These genes were introduced into maize line MON810 via microprojectile bombardment transformation, wherein microscopic beads of gold are coated with DNA and physically forced into maize cells such that the DNA can integrate with the maize DNA/chromosomes in the nucleus. The two plasmid vectors were introduced by microprojectile bombardment into cultured plant cells. This is a well-characterised procedure that has been used for over a decade for introducing various genes into plant genomes. Southern blot analysis and Mendelian genetics data demonstrated that the introduced gene is stably integrated into the maize genome and stably inherited. Glyphosate-tolerant transformed cells were selected, and then cultured in tissue culture medium for regeneration of whole plants. MON810 Bt-maize was developed for control of various insect pest species that cause serious pest problems in maize: corn earworm (CEW), European corn borer (ECB) and Southwestern corn borer (SWCB). These insect species feed on maize, causing plant damage that ultimately results in decreased quality and quantity of yields. During field testing of plants of maize line MON810, ECB infestations were significantly reduced as compared to non-transgenic control plants (AGBIOS, 2004).

In a MON 810 case study, the identity and levels of expression of the Cry1Ab protein in plant tissue samples were determined by ELISA. The Cry1Ab protein levels were low relative to total protein in maize leaf, grain and whole plant tissues, but sufficient to provide season-long control of ECB. The highest levels of protein were found in leaf tissue with 9.35 ug Cry1Ab protein/g fresh weight as compared to 0.31 ug protein/g in grain and 4.15 ug protein/g in the whole plant. The levels of Cry1Ab protein in MON 810 plants is similar when plants are grown in different geographies and when the gene is present in different genetic backgrounds. The level of expression remains consistently high to provide season-long control of the targeted insect pests (AGBIOS, 2004).

1.6 ***Bacillus thuringiensis***

Bt is an insecticidal bacterium, marketed worldwide for control of many important plant pests – mainly caterpillars of the Lepidoptera (butterflies and moths), but also for control of mosquito larvae, and simulid blackflies that vector river blindness in Africa. Bt was first discovered in 1911, as a pathogen of flour moths from the province of Thuringia, Germany. It was first used as a commercial insecticide in France in 1938, and then in the USA in the 1950s. However, these early products were replaced by more effective ones in the 1960s, when various highly pathogenic strains were discovered with particular activity against different types of insect.

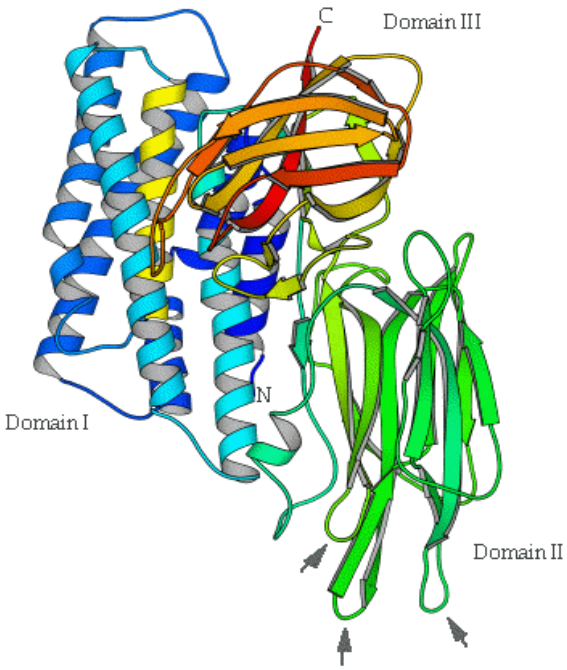
For many years, Bt was available only for control of Lepidoptera, using a highly potent strain (*B. thuringiensis* var. *kurstaki*). This strain still forms the basis of many Bt

formulations. Further screening of a large number of other Bt strains revealed some that are active against larvae of Coleoptera (beetles) or Diptera (small flies, mosquitoes). Most of these strains have the same basic toxin structure, but differ in insect host range, perhaps because of different degrees of binding affinity to the toxin receptors in the insect gut.

The commercial Bt products are powders containing a mixture of dried spores and toxin crystals. They are applied to leaves or other environments where the insect larvae feed. The toxin genes have also been genetically engineered into several crop plants. The crystals are aggregates of a large protein (about 130 – 140 kDa) that is actually a protoxin – it must be activated before it has any effect. The crystal protein is highly insoluble in normal conditions, so it is entirely safe to humans, higher animals and most insects. However, it is solubilised in reducing conditions of high pH (above about pH 9.5) – the conditions commonly found in the mid-gut of lepidopteran larvae. For this reason, Bt is a highly specific insecticidal agent. Once it has been solubilised in the insect gut, the protoxin is cleaved by a gut protease to produce an active toxin of about 60 kDa. This toxin is termed delta-endotoxin. It binds to the midgut epithelial cells, creating pores in the cell membranes and leading to equilibration of ions. As a result, the gut is rapidly immobilised, the epithelial cells lyse, the larva stops feeding, and the gut pH is lowered by equilibration with the blood pH. During natural infection, this lower pH enables the bacterial spores to germinate and the bacterium then invades the host, causing a lethal septicaemia (The Microbial World, 2002).

1.7 **Bt Delta – Endotoxin**

Recent studies on the delta - endotoxin structure show that it has three domains. Domain 1 is a bundle of 7 alpha–helices, some or all of which can insert into the gut cell membrane, creating a pore through which ions can pass freely (The Microbial World, 2002). Domain 2 consists of three antiparallel beta–sheets, similar to the antigen–binding regions of immunoglobulins, suggesting that this domain binds to receptors in the gut. Domain 3 is a tightly packed beta–sandwich that is thought to protect the exposed end of the active toxin, preventing further cleavage by gut proteases (Crickmore and Ellar, 1992).



Cry 3A structure (front view)

Figure 1.1 3-D scheme of the delta – endotoxin from *B. thuringiensis*, depicting the three domains of the protein structure (Crickmore and Ellar, 1992).

B. thuringiensis strains produce two types of toxin. The main types are the Cry (crystal) toxins encoded by different *cry* genes, and this is how different types of Bt are classified. The second types are the Cyt (cytolytic) toxins, which can augment the Cry toxins, enhancing the effectiveness of insect control. Over 50 of the genes that encode the Cry toxins have now been sequenced and enable the toxins to be assigned to more than 15 groups on the basis of sequence similarities.

Since 1996, a wide range of crop plants have been genetically engineered to contain the delta – endotoxin gene from *B. thuringiensis*. These "Bt crops" are now available commercially. These include "Bt maize", "Bt potato" etc. Such plants have been genetically engineered to express part of the active Cry toxin in their tissues, so they kill insects that feed on the crops (The Microbial World, 2002).

The residual effects produced by insecticides, environmental pollution, toxicity and induced resistance in insects provoked the use of microorganisms as an alternative and efficient means of control (Lacey and Harper, 1986).

Lepidopteran pests, particularly the stem borer complex, are a major constraint to increased productivity, and are of economic importance in most maize-growing countries throughout the world. Just under half (46%) of the maize area in the 25 key maize-growing countries have medium (40% area infested in temperate areas) to high levels (60% area infested in tropics/subtropics) of infestation with lepidopteran pests (ISAAA, 2003). Corn rootworm infests 20 million hectares in the Americas, requiring more

insecticide than any other pest in the US, with losses and control measures in the US costing $1 billion per annum. The global losses due to all insect pests is 9%, equivalent to 52 million tons of maize, valued at $5.7 billion annually and consuming insecticide valued at $550 million. Losses associated with lepidopteran pests that can be controlled by Cry1Ab are estimated to cause loses of 4.5%, equivalent to half the total losses from insect pests of maize. Bt maize has proved to be a safe and effective product. Having undergone rigorous testing for food and feed safety, it has provided environmentally-friendly and effective control for targeted pests and the resistance is still durable after seven years of deployment on 43 million hectares. It is the first Bt maize product widely commercialized with proactively implemented, science-based insect resistant management strategies featuring refugia (areas planted to non-Bt maize) combined with high dose technology. Global deployment of the *cry1Ab* gene in Bt maize has the potential to increase maize production by up to 35 million tons valued at $3.7 billion per year: yield gains due to Bt maize are estimated at 5% in the temperate maize growing areas and 10% in the tropical areas, where there are more and overlapping generations of pests leading to higher infestations and losses. From a global perspective the potential for Bt maize in the near to mid-term is substantial. The *cry1Ab* gene has provided effective control of several of the primary pests of maize, principally the stem borers, and intermediate control for other caterpillar pests including armyworm and earworm. The successful performance of Bt maize has resulted in its rapid adoption of 43 million hectares in seven countries, since its introduction in 1996 (ISAAA, 2003). In addition to the significant advantages that Bt maize offers as a pest management tool, the product offers safer feed and food products than conventional maize with lover levels of harmful

mycotoxins, an increasingly important attribute as food and feed safety is assigned higher priority. Of the three major staples, maize, wheat and rice, to-date maize is the only one that offers the significant benefits of commercialized biotechnology. Bt maize now offers an increasing range of options to meet the very diverse needs of the environments in which maize is grown (ISAAA, 2003).

The World Food Programme recently reported that the number of people suffering from malnutrition increased by 25 million from 815 to 840 million. The most compelling case for biotechnology, and more specifically GM crops, is the capability to contribute to a) increasing crop productivity, and thus contribute: to global food, feed and fiber security; b) conserving biodiversity, as a land-saving technology capable of higher productivity; c) more efficient use of external inputs, for a more sustainable agriculture and environment; d) increasing stability of production to lessen suffering during famines due to abiotic and biotic stresses; and e) to the improvement of economic and social benefits and the alleviation of abject poverty in developing countries (James, 2003).

The experience of the first eight years, 1996 to 2003, during which a cumulative total of over 300 million hectares (approximately 750 million acres, equivalent to almost one-third of the total land area of the US or China) of GM crops were planted globally in 21 countries, has met the expectations of millions of large and small farmers in both industrial and developing countries. In 2003, coincidental with evidential confirmation that commercialized GM crops continue to deliver significant economic, environmental, and social benefits to both small and large farmers in developing and industrial countries,

the global area of transgenic crops continued to grow at an annual sustained double-digit growth rate of more than 10%. The number of farmers that benefited from GM crops continued to grow and reached 7 million in 2003, up from 6 million in 2002. Notably, more than 85% of these 7 million farmers benefiting from GM crops in 2003 were resource-poor farmers planting Bt cotton, mainly in nine provinces in China and also in the Makhathini Flats in KwaZulu Natal province in South Africa (James, 2003).

A major concern is the development of insect resistance to Bt. Insects have the potential to develop resistance to insecticides. Based on laboratory selection experiments and models derived from these studies, the potential exists for development of resistance to Bt when these products are overused or used incorrectly. Insects that develop resistance to Bt most commonly exhibit decreased or altered receptor binding, although altered proteolytic activation has also been reported. Stability, persistence and uniformity of coverage are major factors in determining the probability that insects will develop resistance to Bt (Nester, *et al.,* 2002).

1.8 **Methods of Detection of the Bt Trait in Maize**

Since the incorporation of Bt genes into maize and various other crops, various techniques have been adapted for the detection and expression of these genes in crops. A major difficulty with working with transgenic plants is gene silencing. While transgenes are often inherited in a predicable manner, the expression of the transgene often varies in different lines due to position effects or epigenetic effects. Thus, it is necessary to

monitor inheritance and expression of the transgene each generation, to verify that the transgene functions properly in the conditions tested. Inexpensive, high – throughput methods are needed to meet the demand of breeding programmes for screening large numbers of plants (Sangtong *et al*., 2001).

In order to verify claims for enforcement purposes, the common way is by laboratory analysis. In the case of GM – foods, the analytical methods are not totally reliable, not available or very expensive. There is currently only one nucleic acid based technique (PCR) and two immuno-chemical approaches (ELISA and lateral flow) available. PCR has released a whole reservoir of information in evolutionary biology. With its capacity to deal with small amounts of poor quality DNA, the technique has enabled workers to analyse various types of samples (Walker and Gingold, 1993). It can provide discrimination between different transformation events. However, the low limit of detection necessitates stringent material handling and laboratory practices to avoid false results. It is very expensive and has limited quantification capabilities. It is also highly variable. Therefore, it is not reliable at very low target template levels. There is a lack of validation at this stage, a lack of agreement on standardised reference material and it requires extensive training of operators. It takes about three to six hours to complete a PCR assay. The ELISA tests are more economical than PCR, can reliably quantify proteins and are less prone to false positives from minor contamination than PCR. However, development costs of pure proteins and antibodies are high, tests cannot discriminate between different events and significant training is required. Lateral Flow Strips is a technique which is less expensive than PCR or ELISA, it is fast and not much

training is required. It uses a double antibody sandwich format. Antibodies specific to the Cry1Ab protein are coupled to a colour reagent and incorporated into the lateral flow strip. When the lateral flow strip is placed in a small amount of an extract from plant tissue that contains Cry1Ab protein, binding occurs between the coupled antibody and the protein. A sandwich is formed with some, but not all the antibody that is coupled to the colour reagent. The presence of only one line on the membrane indicates a negative sample and the presence of two lines indicates a positive sample (Strategic Diagnostics Inc., 2003). However, high up-front cost for the development of the tests is required, they are qualitative only, cannot discriminate between different events with the same protein and the limit of detection is not as low as ELISA in some cases. Currently, there is a lack of statistically valid sampling methodology. This complicates the situation as both detection ways are indirect approaches in that they measure specific nucleic acid or proteins, the quantity of which is not necessarily related to the percentage GMO in a shipment. Especially commodities that are transferred would be very difficult to sample and analyse for the presence of GMOs or its proteins. There are technical and economical problems associated with testing GMOs in bulk. To make quantitative estimates based on qualitative tests (Lateral Flow Strips) requires multiple statistically valid samples and multiple tests. These are time consuming and very expensive. Testing may need to be repeated at different points as grain moves through the handling and distribution processes, adding additional costs and delays. Method validation is critical to ensure that the tests detect the specific introduced DNA or protein. Only an ELISA and PCR method for Roundup Ready soybean and a PCR for Bt176 and Bt11 maize have been validated up until now. This was done in Europe. South African laboratories will have to take part

in validation schemes in order to be accredited. The conclusion is that the methods of sampling especially of large quantities and in particular analytical methods are not completely acceptable to give reliable results (South Africa, 2001)

1.9 **Half – Seed Extraction Technique**

A half – seed extraction technique, that allows detection of transgenes and their protein products in the endosperm of individual maize kernels, has been developed at the Department of Agronomy of the Iowa State University. The embryo is not damaged so that the kernels can be analyzed, stored, and subsequently germinated. Nonlethal sampling methods that retain seed viability are frequently used to screen kernel traits (Sangtong *et al*., 2001).

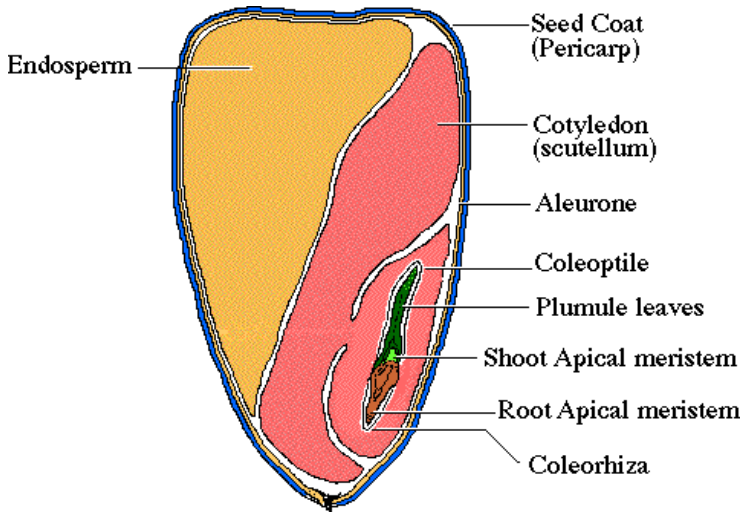


Figure 1.2 Diagram of a cross section of a maize kernel (Earthlink, 2005).

1.10 **Enzyme Linked Immunosorbent Assay**

Immunobinding assays are solid phase assays using immobilised antigen for assessing the antibody content of samples. Enzyme immunometric assays are also often called ELISAs, and to try to avoid confusion, the immunometric version is sometimes called two-site ELISA whereas the binding assay type is known as one-site ELISA. As antigen is simply captured onto tube or well surfaces by non-specific binding, such assays are occasionally known as sticky plate assays or (especially when complex impure antigen solution is used) as dirty plate techniques. They are often regarded as immunoassays, although their value for accurate quantification of antibody concentration can be questioned (if accuracy is important it is usually better to use an immunometric or competitive binding assay). However, immunobinding assays are very easy, quick, cheap and simple, and are ideal for checking the comparative antibody content of sera and other biological fluids and especially for screening sera from immunized animals, hybridoma culture supernatants, ascitic fluid and pathological samples. Antigen-containing solution is simply incubated in plastic tubes or (more often) in the wells of plastic microtitre plates, which allows a (small) proportion of the protein to coat the surfaces of the tubes or wells. After unbound antigen(s) has been washed away, the samples of known or unknown antibody content are incubated in the antigen-coated tubes/wells. Antibody (if present) binds to the immobilised antigen(s) and, after washing, can be detected using labeled anti-immunoglobulin or immunoglobulin-binding protein. Such assays, which use radiolabelled antibody or antibody-binding protein, are normally called solid-phase radiobinding assays, but most assays used nowadays employ enzyme-labelled detecting

reagent. Quantification can be achieved by comparison with a standard solution of known antibody content, but this can be difficult largely due to the heterogeneity of immunoglobulin molecules present (Thorpe and Thorpe, 2000).

1.11 **Polymerase Chain Reaction**

The polymerase chain reaction (PCR) is a method of nucleic acid synthesis, whereby a particular segment of DNA can be specifically amplified. This technique has proved to be an ingenious tool for molecular biology, as it has proved to have more impact on research than restriction enzymes and Southern blotting. PCR is significantly more sensitive and does not always require fresh or properly frozen samples. The principle of PCR is based on two oligonucleotide primers that flank the DNA fragment to be amplified. This allows for the primers to hybridize to opposite strands of the target sequence and is arranged so that the DNA synthesis by the polymerase proceeds across the region between primers. The entire process involves heat denaturation of the DNA, annealing of the primers to their complementary sequence, and extension of the annealed primers with DNA polymerase, repeating those cycles several times to achieve amplification (Clark, 1997).

Real-time PCR and RT-PCR are highly sensitive techniques enabling amplification and quantification of a specific nucleic acid sequence with detection of the PCR product in real time. Quantification of DNA, cDNA, or RNA targets can be easily achieved by determination of the cycle when the PCR product can first be detected. This is in contrast with endpoint detection in conventional PCR, which does not enable accurate

quantification of nucleic acids. Real-time PCR is highly suited for a wide range of applications, such as gene expression analysis, determination of viral load, detection of genetically modified organisms (GMOs), SNP genotyping, and allelic discrimination.

Real-time PCR and RT-PCR allow accurate quantification of starting amounts of DNA, cDNA, or RNA targets. Fluorescence is measured during each cycle, which greatly increases the dynamic range of the reaction, since the amount of fluorescence is proportional to the amount of PCR product. PCR products can be detected using either fluorescent dyes that bind to double-stranded DNA or fluorescently labeled sequence-specific probes.

TaqMan® probes are sequence-specific oligonucleotide probes carrying a fluorophore and a quencher dye. The fluorophore is attached at the 5’ end of the probe and the quencher dye is located at the 3’ end. During the combined annealing/extension phase of PCR, the probe is cleaved by the 5’ → 3’ exonuclease activity of *Taq* DNA polymerase, separating the fluorophore and the quencher dyes. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

The wide variety of fluorescent dyes available makes real-time multiplex PCR possible, providing the dyes are compatible with the excitation and detection criteria of the real-time cycler used. The emission spectra of the chosen dyes must also be sufficiently distinct from one another (Qiagen, 2004).

1.12 **Isoelectric Focusing**

Isoelectric focusing is an end point method. This means, that the pattern – once the proteins have reached their pI's – is stable without time limit. Because of the focusing effect sharp protein zones and a high resolution are obtained. Isoelectric focusing is employed with great success for protein isolation of a preparative scale. It is, however, mainly used for the identification of genetic variations and to investigate chemical, physical and biological influences on proteins, enzymes and hormones.

The use of isoelectric focusing is limited to molecules which can be either positively or negatively charged. Proteins, enzymes and peptides are such amphoteric molecules. The net charge of a protein is the sum of all negative and positive charges of the amino acid side chains, but the three-dimensional configuration of the protein also plays a role.

When a mixture of proteins is applied at a point in a pH gradient, the different proteins have a different net charge at this pH value. The positively charged proteins migrate towards the cathode, the negatively charged towards the anode, until the reach the pH value, where they are isoelectric (Westermeier, 2001).

1.13 **Genetically Modified crops and Human Health**

Most evidence shows that gene transfer using recombinant DNA techniques is substantially different from the processes that govern gene transfer in traditional breeding. In the latter endeavor, plant breeders develop new varieties through the process

of selection and seek to achieve expression of genetic material which is already present within a species. Conventional crossing involves the movement of clusters of functionally linked genes, primarily between similar chromosomes, and includes the relevant promoters, regulatory sequences, and associated genes involved in the coordinated expression of the character of interest in the plant. Genetic engineering works primarily through insertion of genetic material usually from unprecedented sources – genetic material from species, families and even kingdoms that could not previously be sources of genetic material for a particular species. The process involves a 'gene gun', a 'promoter' gene from a virus, and a marker as part of the package or construct inserted into the host plant cell. Current recombinant DNA technologies involve the random insertion of genes in the absence of normal promoter sequences and associated regulatory genes (Altieri, 2001).

1.14 **Molecular Markers**

Genetic markers are any characters that can be measured in organisms, which provides information on the genotype of those organisms. A genetic marker may be a recognizable phenotypic trait or a molecular trait. Whereas phenotypic markers depend on expression of genes and are limited to those genes expressed at a particular time or under particular developmental or environmental conditions, DNA-based markers provide an almost unlimited supply of markers that identify specific sequences across the genome. Their advantages are:

a) single base changes in DNA can be identified, proving many potential marker sites across a genome;
b) they are independent of developmental stage, environment or expression;
c) markers can be found in non-coding or repetitive sequences; and
d) most DNA marker sequences are selectively neutral.

1.15 **Types of Molecular Markers**

There are many potential approaches to identify molecular markers. Most are based on using the polyemerase chain reaction (PCR) to amplify specific DNA sequences. They include:

a) RFLPs (restriction fragment length polymorphisms);
b) RAPD-PCR (random amplified polymorphic DNA);
c) microsatellites or simple sequence repeats (SSRs); and
d) AFLP (amplified fragment length polymorphisms (Walker and Rapley, 2000).

1.16 **Applications of Transgenic Technologies**

The application of transgenic technologies in plant biotechnology has the potential to exceed the technical advances that have taken place in previous ‘revolutions’ in production agriculture. It has only taken four years of commercial growth of transgenic crops in North America for 52% of the soybean, 30% of the maize and 9% of both cotton and canola grown in 1999 to be transgenic, with increasing production of a wide range of

other transgenic crops such as rice, wheat, barley, sorghum, sugar cane, sugar beet, tomato etc. (Walker and Rapley, 2000).

1.17 **Resistance to Bt Crops**

It has been reported that several lepidopteran species have developed resistance to Bt toxin in both field and laboratory tests, suggesting that the major resistance problems are likely to develop in Bt crops, which through the continuous expression of the toxin create a strong selection pressure. In order to delay the inevitable development of resistance by insects to Bt crops, bioengineers are preparing resistance management plans, which consists of patchworks of transgenic and nontransgenic to delay the evolution of resistance by providing susceptible insects for mating with resistance insects (Altieri, 2001)

1.18 **Ecological Concerns**

The ecological effects of genetically engineered crops are not limited to pest resistance and the creation of new weeds or virus strains. Transgenic crops can produce environmental toxins that move through the food chain and also may end up in the soil and water, affecting invertebrates and probably ecological processes such as nutrient cycling. Moreover, the large-scale landscape homogenization with transgenic crops will exacerbate the ecological vulnerability already associated with monoculture agriculture (Altieri, 2001). Farmers that face the greatest risk from the development of insect

resistance to Bt are organic farmers who grow corn without agrochemicals. Once resistance appears in insect populations, organic farmers will not be able to use *Bacillus thuringiensis* in its microbial insecticide form to control the lepidopteran pests. In addition, genetic pollution of organic crops resulting from gene flow (pollen) from transgenic crops can jeopardize the certification of organic crops and organic farmers may lose premium markets. From the history of agriculture it has been observed that plant diseases, insect pests, and weeds become more severe with the development of monocultures, and that intensively managed and genetically manipulated crops soon lose genetic diversity (Altieri, 2001).

1.19 **Consumption of transgenic crops**

The premature commercial release of transgenic crops due to commercial pressures and lax FDA and EPA policies has occurred in the context of a regulatory framework that seems inadequate, nontransparent and in some cases completely absent. In fact, approval for commercial release of transgenic crops is based on scientific information provided voluntarily by companies producing GE crops (Altieri, 2001)

It is estimated that about 50 percent of the corn and soybean based food in the US comes from GE corn and soybeans. Most consumers are not aware of this and have no possibility of identifying transgenic food not labeled as such. Because of the unusual methods used to breed GE crops, some fear that the genetic variants produced could introduce foreign substances into the food supply with unanticipated negative effects on

human health. One major concern is the small but real chance that genetic engineering may transfer new and unidentified proteins into food, triggering allergic reactions in millions of consumers who are sensitive to allergens and have no way of identifying or protecting themselves from offending foods. Biotechnology introduces genes into various plants that are sources of food and food components. Introduced traits include insect and virus resistance, herbicide tolerance and changes in composition or nutritional content. Given such a diversity of traits, there is a potential for allergenic proteins to be introduced into foods from sources with no history of having allergens or that have amino acid sequence similarities to known food allergens (Altieri, 2001)

1.20 **Legislation**

South Africa has legislation in place to assess the safety and risk of GMOs to humans, animals and to the environment. The monitoring of GMOs and products is executed under various existing legislation and legislation currently being developed. Insect resistant maize (MON 810, *cry1Ab* gene) which is a Bt maize has been released and is available on the South Africa market. The EU has passed this maize variety for commodity import. Labelling regulations developed by the Department of Health have been published for comments. Regulations are based on Codex Alimentarius principles for labelling. The South African Draft Regulations propose a set of mandatory requirements, including changes in nutritional value and natural toxins, specific allergens and the presence of human or animal genes or products thereof. Voluntary labelling is suggested for claims of non-GM food that allows for the presence of 1% of adventitious

or technically included GMOs. A non-GM claim has to be verified by an accredited body and compliance with requirements of identity preservation is a prerequisite. Genetically improved or enhanced characteristics may also be voluntary claimed. GM-free labeling is not allowed as GM-free means a zero tolerance, which is practically not possible (South Africa, 2001).

1.21 **Ethical, Social and Economic Considerations**

There is a whole range of ethical, social and economic considerations linked to the genetic modification of foods. Some are more important to certain groups in society than others. For example, different religious groups have different attitudes to the transfer of animal genes to other animals; vegetarians are concerned with the transfer of animal genes to plants; while the transfer of human genes into animals concerns many people from diverse backgrounds. There are also serious questions over the welfare of genetically modified animals. Others object strongly to the notion of patenting life forms, as well as to the whole notion of 'playing God'.

The Nuffield Council on Bioethics (1999), sets out three main principles against which the ethical evaluation of policies or practices can take place. These are as follows:

- The principle of general welfare. This obliges governments and other powerful institutions to promote and protect the interests of citizens, for example by ensuring them access to safe and nutritious foodstuffs, protection from environmental harms, etc.;

- The ability of people to claim their rights, including their freedom of choice as consumers. However, the ability of people to claim their rights in turn imposes obligations, for example on governments, producers or suppliers and the rights of different groups often compete – consumers may have the right not to consume genetically modified foods, but they will be unable to claim this right unless there are labelling rules to inform their choice; and
- The principle of justice. This requires the burdens and benefits of policies and practices to be fairly shared among all those who are affected by them.

The distinction between ethical and social considerations is a fine one. The Council points out that 'on the one hand, ethical principles concern the social framework within which we live, on the other, we need to be aware of the social and technological background against which we discuss ethical issues.' Especially difficult to resolve, the Council claims, are questions hinging on the ethical status of the natural world itself. While 'GM crops do not raise questions about the rights or welfare of plants… they do prove a reaction that is difficult to place within arguments about welfare, rights and justice.' (Nuffield Council on Bioethics, 1999)

1.22 **The Scope of the Present Study**

The development of transgenic plants resulted in the need to utilize the various molecular methods (e.g., ELISA, real - time PCR etc.) for the detection or analysis of the presence or absence of a specific trait in a particular plant (Bt in this study). The overall aim of this

study was to optimize a half – seed extraction technique as part of a laboratory protocol for transgenic maize plants and to explore the possibility of using the following molecular techniques: horizontal isoelectric focusing, real - time PCR and ELISA, as methods for detection of the Bt trait for incorporation into the half – seed extraction protocol.

In order to satisfy the above aim, the following objectives for the study were formulated:

1.22.1 to isolate storage proteins and DNA from maize seed using a half – seed extraction technique;

1.22.2 to analyse extracted proteins for the expression of Bt genes using IEF gel electrophoresis and ELISA;

1.22.3 to analyse extracted DNA for the presence of Bt transgenes using real time–PCR;

1.22.4 to optimise electrophoresis for its utilisation as a cost - effective routine screening technique for Bt maize; and

1.22.5 to determine the relationship between gene inheritance and gene expression by comparative analysis of PCR and expression results.

CHAPTER 2: MATERIALS AND METHODS

2.1 **Half – seed Extraction Technique**

The half – seed extraction technique was used for both storage protein and DNA isolation. The sample material used for the storage protein and DNA isolation were four maize lines [Line A (Non Bt), Line B (Bt), Line C (Non Bt) and Line D (Bt)] and two maize hybrids [Hybrid A (Non Bt) and Hybrid B (Bt)]. Ten kernels of each sample were tested and all analyses were performed in triplicate. Seeds were soaked in distilled water for approximately 24 hours. Approximately 100 mg of each kernel was removed without damaging the embryo of the kernel and the fraction was placed in a 48 – well cell culture cluster (Costar). The remainder of the kernel was coated with gently - heated leg wax to prevent water from entering the point of incision that could cause the seed to rot or get infected, thereby preventing germination. The coated kernels were germinated in soil in a hot house for approximately two weeks. The extracted storage protein was used for analysis on IEF. IEF analysis involved various gel types (Proteios) that had specific ampholytes incorporated into them to allow one to have various degrees of protein separation based on a specific pI range in a gel. Upon germination, two leaf punches were taken from each plant and protein was extraction was done for the analysis on ELISA.

2.1.1 Reproducibility, repeatability and validity of half - seed technique

In order to establish whether the developed protocol was consistently reproducible and repeatable, samples groups were germinated after protein and DNA extraction in triplicate. Extracted protein and DNA samples were subjected to electrophoresis, ELISA and PCR analysis.

2.2 **Protein Extraction from Seed Fraction for Isoelectric Focusing**

Protein extraction buffer (0.01M Tris – citric acid pH 7.0) was added to the 100 mg seed fraction that was removed in a 1:1 ratio (w/v). The seed fraction was crushed using an adapted drill bit (Proteios) for a bench top drill press (Ryobi) that fits in the well of a 48 - well cell culture cluster. The entire volume of extracted protein was pipetted into a micro centrifuge tube (Plastibrand) and stored in an ultra freezer (Nuare) at - 84ºC until electrophoresis were performed. Seed residue was stored at - 84ºC for DNA extraction.

Table 2.1 Optimization of protein extraction using various buffers, gel types and stains for the potential isolation of the Bt endotoxin

Gel Type	Buffers used	Stains
Mai001	Tris – citric acid pH 8.0 Trypsin Extraction Carbonate Western Blot Phosphate	Coomassie/ Silver
Sun005	Tris – citric acid pH 8.0 Trypsin Extraction Carbonate Western Blot Phosphate	Coomassie
Tom002	Tris – formic acid pH 8.0	Malic Dehydrogenase
Tom007	Double distilled water	Esterase Alcohol Dehydrogenase

2.3 **Isoelectric Focusing**

2.3.1 Prefocusing

A 20 X 30 cm precast polyacrylamide (PAG) gel (Proteios) was placed on a 10ºC cooled electrophoresis bed (Multiphor II electrophoresis system). Two wicks (Proteios ProtWicks) were saturated with an anode buffer (25.5 mM L - aspartic acid, 24.5 mM L – glutamic acid in distilled water) and upon removal of excess buffer placed on the outer most ends of the PAG gel. A single wick (Proteios ProtWicks) was saturated with cathode buffer (25.2 mM L – arginine, 24.6 mM L – lysine, 12% ethylenediamine in distilled water) and upon removal of excess buffer placed in the middle of the two anode

wicks. Each of three electrodes from the electrophoresis system was placed on the respective wicks. Upon connection, the PAG gel was prefocused at 200 V, 30 W and 12 mA for 100 volt hours (approx. 20 minutes) using a volt hour integrated electrophoresis power supply (EPS3501 – XL) (Proteios, 2001).

2.3.2 Electrophoresis

The lid of the electrophoresis unit as well as the electrodes were removed and placed aside. The electrodes were wiped with tissue to prevent the buffers from drying on it and forming crystals that could interfere with the run. A 52 - well loading chamber (Hypure) was placed 3 – 5 mm from each anode as per electrophoresis protocol. 22 uL of the extracted storage protein (unknown concentration) was loaded in each well. The electrodes and the lid of the electrophoresis unit were replaced. A gel entry step was run at 200 V, 30 W and 12 mA for 100 volt hours (20 min.) and the gel was focused for 1500 volt hours (80 min.) (Proteios, 2001). For optimization purposes, Bt samples were run next to their respective non-Bt counterparts as controls. For screening purposes, each line and hybrid group were run individually to determine genetic purity for quality control purposes only. During optimization, protein standards (i.e., with known pI) were not used.

2.3.3 Fixing

Upon completion of electrophoresis, the electrode wicks and the 48 - well loading chambers were removed and the gel was placed in a glass staining tray (Pyrex) with 250 mL of a 20% trichloroacetic acid solution. The gel was allowed to fix unshaken for 15 min. The gel was then allowed to fix for another 15 min on an MRC orbital shaker at 100 rpm (Proteios, 2001).

2.3.4 Staining

Upon completion of protein fixing, the fix solution was discarded and the gel was rinsed with 250 mL distilled water. 250 mL Coomassie blue stain [0.1% Coomassie Brilliant Blue R (Sigma) dissolved in destain solution (1 volume glacial acetic acid, 4 volumes methanol, 5 volumes double distilled water)] which was gently poured over the gel and placed on the orbital shaker to stain until the protein bands reached a deep blue intensity. The Coomassie blue stain solution was discarded and the gel was rinsed with distilled water. The gel was destained with 250 mL destaining solution until the background gel was clear and only the protein banding pattern visible. The destain solution was discarded and the gel rinsed with distilled water. The gel was removed from the water and allowed to dry at 37ºC in an incubator (Proteios, 2001).

2.3.5 Analysis of IEF Protein Banding Patterns

The dried gel was annotated and visually scored on a light box. Scoring was done by comparing the protein profile of each kernel to each other in a specific group. Any extra protein band or deletion of a protein band was considered as a non conformance and therefore regarded as an off – type. Genetic purity was calculated as a percentage, as discussed above. A percentage of 96% and more were regarded as the sample having passed (Proteios, 2001).

2.4 **DNA Analysis**

2.4.1 Optimization of DNA extraction

DNA extraction was optimized on maize kernels using a CTAB method (ISTA/FAO, 2003) and the GenElute Plant Genomic DNA Miniprep Kit (Sigma, 2006). Subsequent analyses were done using the CTAB method.

2.4.2 DNA extraction from seed fraction

DNA was extracted from the seed residue after the storage protein extract was removed. A seed extraction solution (20 g/L CTAB [Sigma], 1.4 M NaCl, 0.1 M Tris/HCL, 20 mM EDTA) was added to the protein extraction residue in a 1:1 (w/v) ratio. The mixture was incubated at 65ºC for 45 min. and shaken gently every 5 min. to lyse the plant cell wall

and liberate DNA. The tubes were centrifuged at 12 000 *g* for 10 min. The aqueous phase was transferred into a new 1.5 mL eppendorf tube and an equal volume of chloroform was added. The tubes were mixed by inversion for 30 seconds and centrifuged for 10 min. at 12 000 *g*. The upper aqueous phase was transferred into a new 1.5 L eppendorf tube and two volumes of CTAB precipitation buffer (5 g/L CTAB, 0.04 M NaCl) was added and incubated at room temperature for 1 hour. After incubation, the tubes were centrifuged at 12 000 *g* for 5 min. The supernatant was discarded and the precipitate was resuspended in 350 uL 1.2 M NaCl. 350 uL of chloroform was added to remove contaminating proteins. The tubes were inverted to mix the solutions and centrifuged at 12 000 *g* for 10 min. The supernatant was carefully removed and two volumes of 100% isopropanol were added in clean eppendorf tubes. The DNA was allowed to precipitate in a freezer at –20°C overnight. The pellet was centrifuged at 12 000 *g* for 10 min and the supernatant discarded. The pellet was washed with 70% ethanol and centrifuged at 12 000 *g* for 10 min. The supernatant was removed and the pellet was allowed to air dry and resuspended in 30 uL distilled water (International Seed Testing Association [ISTA], 2003).

2.4.3 DNA concentration determination

The concentration of the extracted DNA was estimated by agarose gel electrophoresis as it allowed one to not only determine the concentration of the extracted DNA but also allowed on to look at the quality of the DNA that has been extracted. Agarose gel electrophoresis was performed on a 0.7% agarose gel [0.7 g agarose (BIOLINE),

0.0001% ethidium bromide in 100 mL 1X TBE pH 8.3 solution [10.8 g (Trishydroxymethyl), 5.5 g boric acid, 0.93 g EDTA up to 1L with double distilled water)]. Three uL of the dissolved DNA was added to 2 uL of gel loading buffer (30% w/v glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol FF [Sigma] dissolved in distilled water) and loaded in a 0.7% agarose gel. Three uL of control phage lambda DNA (Promega) with known concentration of 20 ng/uL was loaded on either side of the sample. The gel was allowed to run at constant voltage of 75 Volts for 30 – 45 min. With the migration of the DNA through the gel matrix, the DNA bound with the ethidium bromide in the gel which caused the DNA to fluoresce under ultraviolet light. The gel was viewed using a Dual Intensity Ultraviolet Transilluminator (UVP) that was linked to a camera (UVP). The entire UVP system was linked to a standard desktop computer. Grab – it (UVP) software was used for visualization and capture. The DNA concentration was estimated by comparison to the control using the Genetools computer programme. The programme allows one to compare the intensity of the fluorescence of the standard with known concentration to that of the DNA. Based on this comparison, a reading was given with an approximation of the DNA concentration.

2.5 **TaqMan Chemistry for Determining Homozygosity in Transgenic Plants**

The TaqMan method exploits the 5′ – 3′ nuclease activity of AmpliTaq Gold DNA Polymerase to allow direct detection of the PCR product be the release of the fluorescent reporter during PCR amplification (ABI PRISM 7000 Sequence Detection System, 2001).

In PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The target sequence is then amplified. The TaqMan probe hybridizes to a target sequence within the PCR product. When the PCR product is amplified in subsequent cycles, Amplitaq Gold DNA Polymerase cleaves the Taqman probe (5′ – 3′ nuclease activity) so that the enzyme can continue to copy its target sequence. The reporter dye and quencher dye are separated, resulting in increased fluorescence of the reporter (ABI PRISM 7000 Sequence Detection System, 2001)

2.5.1 PCR reaction setup

2.5.1.1 Preparing control and sample reactions

The amount of GMO PCR cocktail needed for all of the samples to be analysed was calculated. Each sample required 44 uL of TaqMan GMO mix (Applied Biosystems) and 1 uL of Amplitaq Gold DNA Polymerase. The cocktail mixture was vortexed for 10 seconds. After annotating a MicroAmp Optical 96 – well reaction plate, 45 uL of the prepared cocktail was pipetted into all of the wells that were to be used. 5 uL of the sample DNA preparations were pipetted into each of the sample wells. 5 uL of the negative control (FLUKA maize DNA standards) were pipetted into each of two negative control wells. 5 uL of the positive control (FLUKA maize DNA standards) were pipetted into each of two positive control wells (ABI PRISM 7000 Sequence Detection System, 2001).

2.5.2 PCR thermal cycling

The PCR thermal cycle consisted of three stages. An initial stage at 50ºC for 2 min. repeated once, A second stage at 95ºC for 10 min repeated once, and a third stage that encompassed two settings of 95ºC for 5 sec. and 60ºC for 1 min., repeated 40 times (ABI PRISM 7000 Sequence Detection System, 2001)

2.5.3 Analysis of PCR results

After running the samples on the instrument, the data was analysed using the ABI PRISM 7000 Sequence Detection System software to produce Ct values of each of two reporter dyes (FAM and VIC) for each sample that was run. The amount of GMO material (FAM) dye was normalized to the amount of plant material (VIC) dye detected in each sample. This produced a delta Ct value which was averaged for replicate samples. These values were compared to a calibration curve produced from the delta Ct values of the known GMO concentration standards. This enabled a %GMO result for each unknown sample to be produced (ABI PRISM 7000 Sequence Detection System, 2001)

2.6 **Enzyme Linked Immunosorbent Assay**

2.6.1 Protein Extraction from Leaf Samples

The same lines and hybrids that were used for storage protein extraction and DNA extraction and subsequently germinated, were used for the ELISA analysis. A double leaf punch using the lid of the eppendorf tube for sampling, were taken from the two week old maize seedlings. 500 uL of a phosphate buffered saline (PBS) (80g sodium chloride, 0.027 M potassium chloride, 0.08 M anhydrous di–sodium hydrogen orthophosphate, 0.015 M potassium dihydrogen orthophosphate dissolved in distilled water) was added to the double leaf punch. The samples were ground using a bench top drill and an adapted drill bit. Upon homogenization, tubes were centrifuged at 12 000 *g* for 10 min. For the assay, the supernatant was recovered and diluted in a dilution of 1:11 parts with 1 X PBS (Envirologix, 2005).

2.6.2 Assay

50 uL of a conjugate solution supplied in the ELISA kit (Cry1Ab/Cry1Ac Enzyme Conjugate) was added to a 96-well Cry1Ab/Cry1Ac antibody - coated solid plate supplied in the ELISA kit. A further 50 uL of extracted protein was added immediately to each well and 50 uL of the positive control (Cry1Ab/Cry1Ac) and buffer (PBS) blank to their respective wells. The plate was shaken by gently tapping it against the thumb for 20 – 30 seconds to thoroughly mix the contents of the wells. The plate was covered with

parafilm to prevent evaporation and incubated at room temperature for 1 hour. After incubation, the parafilm was removed and the plate was washed three times with a phosphate buffered saline with 0.55% Tween 20 (PBST) solution, by flooding the wells with the buffer and shaking it out. The plate was dried and excess buffer was removed by tapping the plate vigorously on tissue paper to ensure that there was no residual buffer. 50 uL of a substrate solution (Envirologix Kit) was added and allowed to incubate for 30 min at room temperature. Prior to the addition of 50 uL of a stop solution (1 N hydrochloric acid), a blue colour reaction was observed that turns yellow with the addition of the stop solution. The plate was analysed visually within 30 minutes of the addition of the stop solution (Envirologix, 2005).

2.6.3 Analysis of ELISA results

Each sample was analysed qualitatively by comparing the colour change to the control samples. A bright yellow colour indicated a positive test for the Cry1Ab Bt endotoxin and a blank well indicated a negative test for the same endotoxin (Envirologix, 2005). A sample that tested positive for the Bt trait, did not indicate whether the hybrid was homozygous or heterozygous for the specific trait. Quantitative analysis could have been performed using a spectrophotometer. With quantitative analysis, the optical densities of the sample extracts are compared to those of the positive control. Samples with absorbance close to that of the blank wells are presumed to be free of the Bt endotoxin. Samples with absorbencies significantly higher than those of the blank wells are positive for Bt endotoxin content (Envirologix, 2005).

CHAPTER 3: RESULTS

3.1 **Half – seed Extraction Technique**

Storage proteins of maize in 4 lines and 2 hybrids were analysed using a half-seed extraction protocol in order to preserve the embryo for germination. The technique had to be optimized to establish the various parameters in order to develop a laboratory protocol. Optimization involved determining which extraction buffers to use for protein extraction, imbibing time to enable non destructive incision into the seed and ensure germination and imbibing temperature to prevent premature embryo germination. Also to determine weight to buffer volume ratio. Upon optimization, all subsequent analyses were performed using this method.

3.1.1 Optimization of Half – seed Extraction Technique for Protein and DNA

Figure 3.1 A kernel of maize with approximately 100 mg of storage protein removed after soaking in double distilled water for 24 hours at approximately 21°C.

The enlargened view of Fig. 3.1 depicts a large flat seed (LFS) where a fraction of the endosperm has been removed. This fraction was removed for storage protein extraction as well as DNA extraction. It forms part of the first stage of the half-seed extraction technique. Fractions were removed from mixed grades of seed (i.e. flat, round, medium, small and large). The weight of the fractions ranged from 20 – 50 mg. The exposed section of the seed was covered in leg wax and germinated in steam sterilized soil. The seedlings were germinated for two weeks and leaf material was harvested for ELISA.

3.1.2 Seed Coating with Leg Wax after Fraction Removal

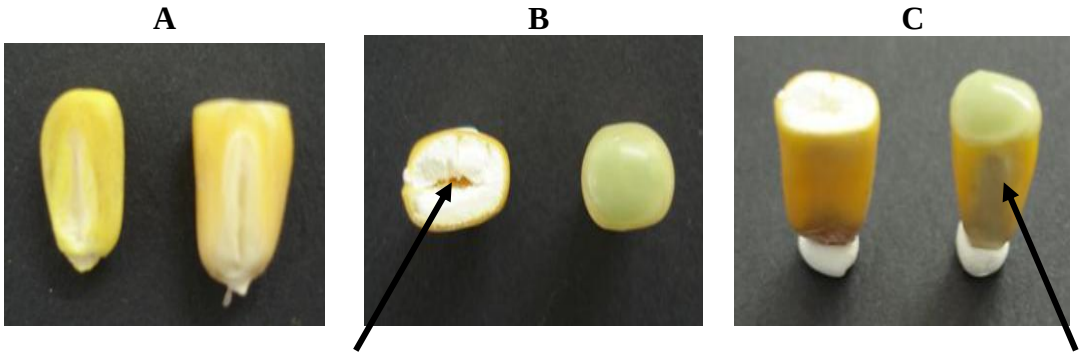


Figure 3.2 Images representing kernels of maize without a wax covering (A and B) and sealed with a wax covering (C) using leg wax melted at 80°C.

Fig 3.2 (A) shows a 2-D view of a large yellow maize seed before (A) and after (B) the seed fraction has been removed. Upon removal, the seed was ready to be covered with leg wax. The fraction was removed after a 24 h imbibing period in double distilled water at 21°C.

Fig 3.2 (B) shows the top view of a maize seed that has been cut to remove a fraction (left) and coated with leg wax (right) to seal the point of incision (light green colour). The

coated seed was then germinated in steam sterilized soil. The unsealed seed has a hole (arrow) where water can enter. In the trials, germination rates were affected negatively due to water entering the point of incision and causing the embryo to rot prior to germination. The exposed point of incision also rendered the seed susceptible to microbial infections that also caused the seed to rot prior to germination thus reducing germination rates.

Fig 3.2 (C) shows a horizontal view of a maize kernel after it has been cut (left) and after it has been sealed off. In this picture, the arrow points to the embryo of the seed and the incision is just above the embryo. In addition, the wax only covers the seed lightly and does not cover the embryo to ensure that the wax did not interfere with germination.

3.1.3 Germination of Maize Kernels

Figure 3.3 Two week old maize seedlings following half – seed technique and germinated in steam sterilized potting soil. Seedlings are at a three leaf stage.

Fig 3.3 shows maize seedlings in a seedling tray that was germinated for two weeks in a hot house. The germination rates were monitored as well as the general condition of the seedlings (i.e. colour, water, heat stress and if fertilizer was required). The second leaf of each plant was harvested at this stage. From the leaf, a double leaf punch was taken with a 1.5 mL eppendorf tube lid. An ELISA extraction buffer was added, the sample was ground and analysed for the presence of Bt endotoxin. Controls, i.e. seed of the same line or hybrid that were not subjected to the incision, were also germinated to compare germination results. Germination results for all the lines and hybrids were high (Table 1).

Table 3.1 Summary of germination results following half – seed technique

Sample Name	Repetitions	Number of kernels tested	Number of kernels germinated	Percentage germination
Line A	1	15	15	100
(Non Bt)	2	15	15	100
	3	15	15	100
Line B	1	15	15	100
(Bt)	2	15	15	100
	3	15	15	100
Line C	1	15	15	100
(Non Bt)	2	15	13	86.66
	3	15	15	100
Line D	1	15	15	100
(Bt)	2	15	15	100
	3	15	13	86.66
Hybrid A	1	15	15	100
(Non Bt)	2	15	12	80
	3	15	11	73.33
Hybrid B	1	15	15	100
(Bt)	2	15	15	100
	3	15	15	100

Germination of 4 lines and 2 hybrids of maize kernels that were used as sample material and were analysed (Table 3.1). Lines A, B and D and hybrid B had 100% germination in all three repetitions. Line C only had 100% germination in reps 1 and 3. Rep 2 had a germination rate below 90%. This however was brought about by over watering which caused some of the seed to rot. The same applied for rep 3 line D. Rep 3 of hybrid A showed the lowest germination. This could have been brought by damage that occurred to the seed during the fraction removal as well as dull seed. The germination rates did not affect the rest of the process as more seed was planted than what was required and enough sample material could be collected. It was found that approximately 20 - 30% extra of the seed should be germinated to ensure enough sample material (Table 3.1).

3.2 **Isoelectric Focusing**

3.2.1 Isoelectric focusing optimization on Mai001 gel type

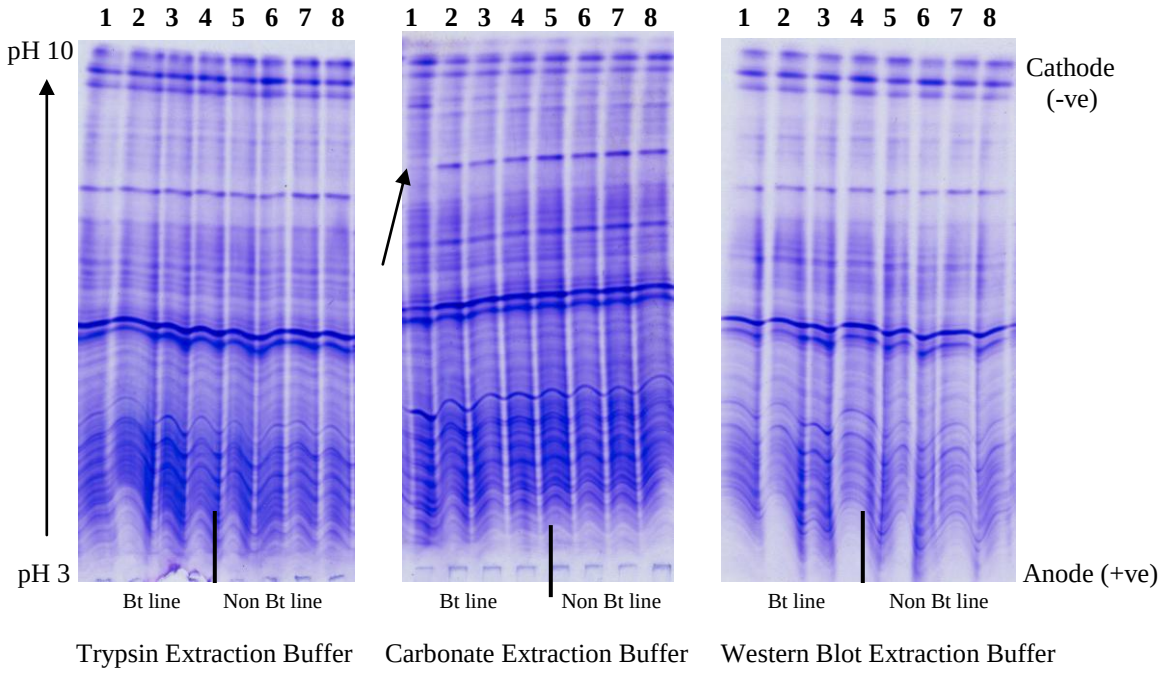


Figure 3.4 Electrophoretic analysis of a Bt and a non Bt line using three extraction Buffers on a Mai001 gel.

Three extraction buffers were used during the optimization process for horizontal IEF gels in order to try and identify the Bt endotoxin (Fig 3.4). On each gel, proteins bands of different sizes were visible where the larger proteins were closer to pH 3 and the smaller proteins separated out to pH 10 focusing at their specific pI. No significant variation between the Bt and non Bt lines are visible in any of the three gels. For the carbonate extraction buffer gel, in lane one there was an absence of the protein band. However, this

could be due to the sample having an off – type in it or it could be segregating. The lower intensity of gel 3 (far right) was due to the cathode being too wet during the IEF run.

3.2.2 Isoelectric focusing optimization on Sun005 gel type

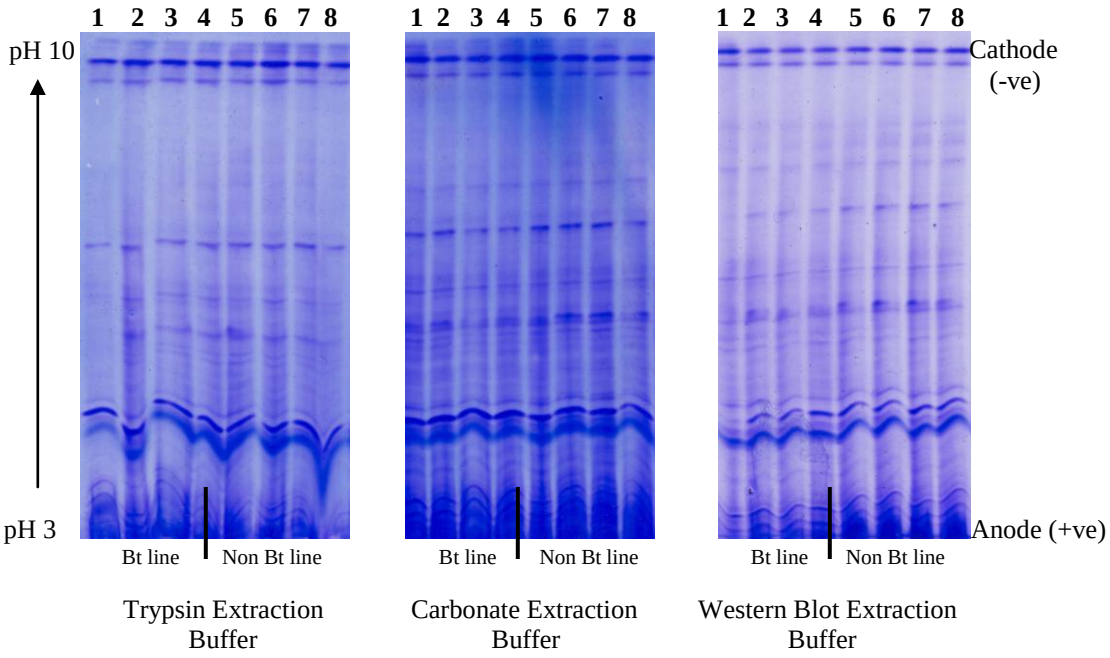


Figure 3.5 Electrophoretic analysis of a Bt and a non Bt line using three extraction Buffers on a Sun005 gel. Protein bands separated over a pI range of 3 – 10 with smaller increments between each pI opening a different window for protein visualization.

Three gels were run on horizontal PAG electrophoresis using three different extraction buffers (Fig 3.5). However, the gel type used was different. This gel enable one to look at more specific pH range and the proteins that fall in that range. This is achieved by adjusting the set of ampholytes used in this specific gel (Sun005). At the base of the gel,

the proteins seem to have a ‘smiling effect’. This was brought about by the fact that the gel was a bit old. The age of the gel is important and it has been noted that fresher gels have sharper focus than that of slightly older gels. No difference between the Bt and non Bt line was detected and neither was an off-type detected in lane one of the carbonate extraction buffer as different samples were analysed on this gel. Coomassie staining was used due to its affordability.

3.2.3 Isoelectric focusing optimization on Tom007 gel type

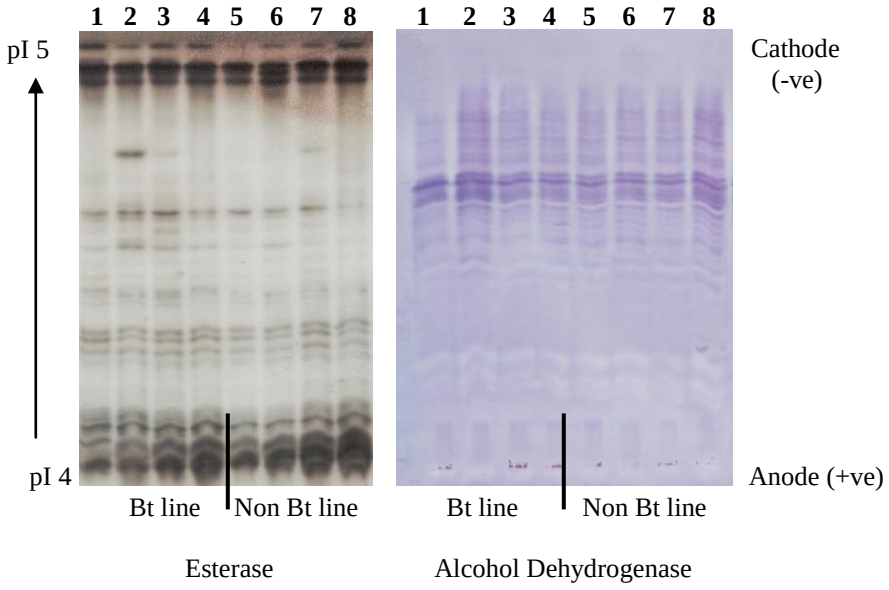


Figure 3.6 Electrophoretic analysis of a Bt and a non Bt line on a polyacrylamide gel (Tom007), stained with Esterase and Alcohol Dehydrogenase (ADH).

Two gels were run on horizontal IEF with isozyme staining (Fig 3.6). Esterase was run on a Tom007 gel and samples were extracted in double distilled water (left). The stain picked up no differences between the Bt and the non Bt line that could have had a

possible link to the expression of the Bt endotoxin. However, there was variation in samples two and 7. The presence of these additional bands could have been due to a number of reasons. These could have been off – types or it could have been segregation in both the lines. However, this was not easy to determine as esterase is generally difficult to map (Proteios, 2006).

ADH (right) was run on the same gel type (Tom007) and proteins were also extracted in water as per the protocol. No differences were picked up between the Bt and non Bt line. The banding pattern was different to that of the esterase stain which allowed one to look at a different set of proteins. From the gel, one could determine that there are no off – types and that the two line are uniform, thus having a good genetic purity.

3.2.4 Isoelectric focusing optimization on Tom002 gel type

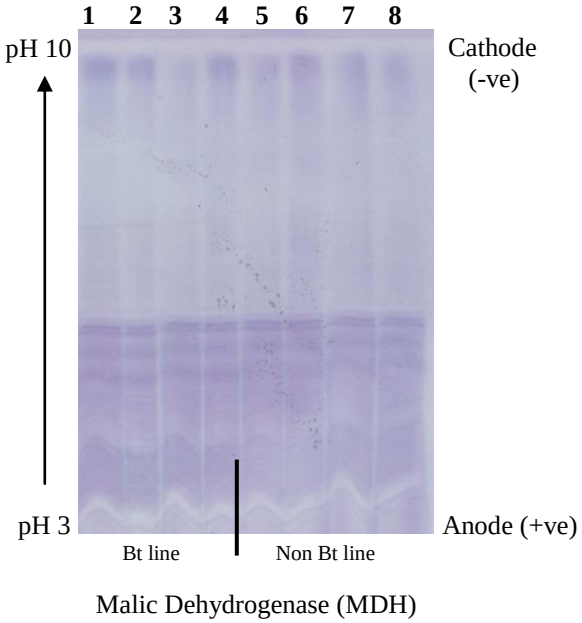


Figure 3.7 Electrophoretic analyses of a Bt and a non Bt line using isozymes on a Tom002 gel in Tris – formic acid pH 8.0 extraction buffer and MDH staining.

A third isozyme was run on IEF during the optimization process (Fig 3.7). The banding pattern is different to that of esterase and ADH. No differences between the Bt and non Bt line was detected. From the banding patterns, it was possible to see the uniformity of the two lines and determine its genetic purity in that there were no off – types.

3.2.5 Isoelectric focusing of storage protein upon optimization of technique

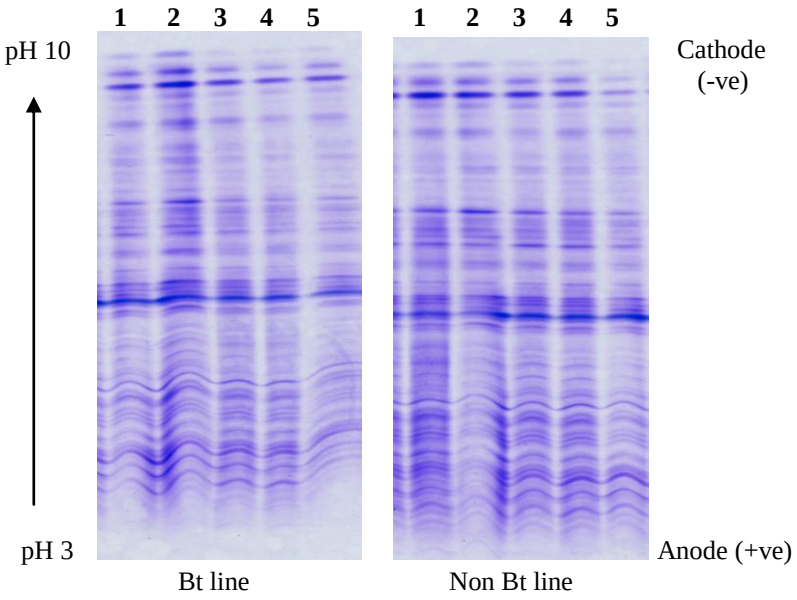


Figure 3.8 Electrophoretic banding pattern of protein extracted from seed portion in a ratio of 1:1 w/v in Tris – Citric acid pH 7.0 and run on horizontal PAG IEF with a pH range of 3 – 10.

After optimization, samples were run on a Mai001 gel for genetic purity (Fig 3.8). There was no variation visible on the gel which indicated that all the samples were uniform. The gel shown was a section of a full gel where 96 samples were run in total. The results are summarized in the table below. A general buffer was used as the Bt endotoxin could not be isolated thus the samples were only screened for genetic purity.

Table 3.2 A summary of electrophoretic analysis of storage proteins analysed by PAG electrophoresis and coomassie and diaphorase enzyme staining

Sample Name	Repetitions	Number of kernels analysed	Total Protein Purity	Isozyme Purity (Diaphorase)
Line A	1	10	100%	100%
(Non Bt)	2	10	100%	100%
	3	10	100%	100%
Line B	1	10	100%	100%
(Bt)	2	10	100%	100%
	3	10	100%	100%
Line C	1	10	100%	100%
(Non Bt)	2	10	100%	100%
	3	10	100%	100%
Line D	1	10	100%	100%
(Bt)	2	10	100%	100%
	3	10	100%	100%
Hybrid A	1	10	100%	100%
(Non Bt)	2	10	100%	100%
	3	10	100%	100%
Hybrid B	1	10	100%	100%
(Bt)	2	10	100%	100%
	3	10	100%	100%

3.3 DNA Analysis

3.3.1 DNA extraction optimization from seed fraction

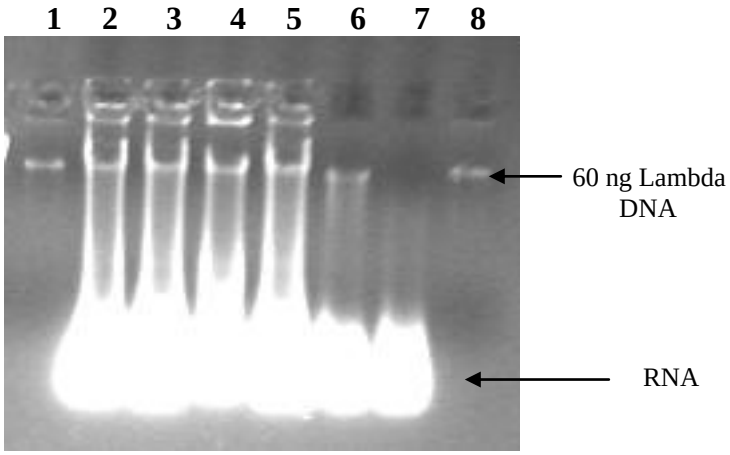


Figure 3.9 Agarose gel electrophoresis of DNA extracted from seed using two DNA extraction protocols for seed. Samples are run in conjunction with a 20 ng/uL Lambda DNA control.

Wells 1 and 8 in Fig 3.9 were two lambda DNA controls that were run in conjunction with samples 2 – 7 on a 0.7 % agarose gel to determine the concentration of samples 2 – 7. The DNA was extracted without the addition of RNase, hence the high fluorescence at the base of the gel. RNase was not used as the RNA did not interfere with the Real-time PCR and would have only increased the cost of analysis. Two methods of extraction were used. Samples 2 – 5 were extracted using a CTAB method developed by the International Seed Testing Association (ISTA). Samples 6 and 7 was extracted using the Genelute DNA purification kit from Sigma. Higher yields of DNA were extracted using the ISTA method as opposed to the Sigma kit. The extractions were performed on whole seed as part of the optimization. The concentration of the DNA extractions on the fraction

removed using the half seed method was very low and sometimes not visible on the agarose gel. For Real - time PCR this proved sufficient as it required only one copy number for the test to work.

3.3.2 Real – Time PCR analysis

Table 3.3 Summary of Real Time – PCR results performed using the ISTA CTAB seed DNA extraction protocol and analysed on the ABI PRISM 7000 sequence detection system

Sample Name	Repetitions	Number of kernels analysed	Percentage Homozygous	Percentage Heterozygous
Line A	1	10	100	-
(Non Bt)	2	10	100	-
	3	10	100	-
Line B	1	10	100	-
(Bt)	2	10	100	-
	3	10	100	-
Line C	1	10	100	-
(Non Bt)	2	10	100	-
	3	10	100	-
Line D	1	10	100	-
(Bt)	2	10	100	-
	3	10	100	-
Hybrid A	1	10	100	-
(Non Bt)	2	10	90	10
	3	10	90	10
Hybrid B	1	10	100	-
(Bt)	2	10	100	-
	3	10	100	-

Analysis of the data from Real-time PCR demonstrated that lines A, B, C, D and hybrid B were 100% homozygous (Table 3.3). However, two of the repetitions of hybrid A showed 10% to be heterozygous for the Bt trait.

3.4 **Enzyme Linked Immunosorbent Assay**

Table 3.4 Summary of ELISA analysis for Bt trait using the Envirologix Kit for Cry1A/Cry1B on leaf.

Sample Name	**Repetitions**	**Number of kernels analysed**	**Percentage positive for Bt trait**	**Percentage Negative for Bt trait**
Line A	1	10	-	100
(Non Bt)	2	10	-	100
	3	10	-	100
Line B	1	10	100	-
(Bt)	2	10	100	-
	3	10	100	-
Line C	1	10	-	100
(Non Bt)	2	10	-	100
	3	10	-	100
Line D	1	10	100	-
(Bt)	2	10	100	-
	3	10	100	-
Hybrid A	1	10	-	100
(Non Bt)	2	10	-	100
	3	10	-	100
Hybrid B	1	10	100	-
(Bt)	2	10	100	-
	3	10	100	-

Use of the Envirologix Kit and visual examination of the colour development in microtitre plates after the ELISA reactions showed that all Bt lines were confirmed as

positive, whereas non-Bt lines and hybrids tested negative (Table 3.4). This occurred for all repetitions for the 10 kernels tested for each type of maize.

CHAPTER 4: DISCUSSION

The study was designed to develop a standard laboratory protocol that implements a half – seed extraction technique for maize seed and encompasses various aspects of molecular screening for a specific trait (presence or absence of the Bt gene/phenotype) that would aid as a tool for breeders in their breeding programs. Various existing molecular techniques were investigated to determine their advantages and disadvantages and how they could be incorporated into a specific protocol by comparing the various aspects of the technique.

In order to ensure that the half – seed extraction protocol was reproducible and repeatable, a number of parameters had to be investigated, in order to ensure the validity of the results. Various steps at each stage of the study were taken to ensure a high level of accuracy, reproducibility and repeatability. These included: completing each set of samples to be analysed in triplicate; optimizing the variables such as buffers for extractions, size of seed fraction removal, imbibing periods for sampling in the half – seed extraction technique, etc.

The literature with regard to this technique did not quite explain the sensitivity of trying to remove a portion of seed without damaging the embryo and without compromising the proteins. The initial attempts involved the removal of part of the seed using a scalpel. However, due to the dryness of the seed, it resulted in cracks that ran through the kernel thus damaging the embryo and preventing germination. Imbibing of the seed seemed to

be the only way to soften the seed. However, factors such as temperature, time of imbibing and preserving the embryo had to be considered. Temperature played an important role because the seeds were imbibed in double distilled water and at too warm temperatures, germination of the seed would occur which would render the seed useless for processing. In addition, if the temperature was too cold, the germination rates were low, due to cold stress on the seed. The time of imbibing also played an important role because the longer the seed remained in the water, the better the chance that germination could take place. The ideal conditions were eventually determined to be an imbibing period of 48 h at 21°C. These conditions proved to soften the seed just enough to cut a fraction off the seed for storage protein isolation and to not affect the seed embryo for germination. Germination was also affected post incision, due to water entering the exposed section of the seed. Water entry caused the seed to rot and decreased the level of germination in the sample groups. In addition, the embryo was exposed to microbial infection. Parameters investigated to reduce infection included the addition of anti – microbial agents added to the soil used for germination and to the irrigation water. Both attempts were unsuccessful. A solution to prevent this contamination was to seal off the exposed area on the kernel. Leg wax at approximately 80°C proved to be the most efficient means to seal the kernel. It was found to be a quick, inexpensive and safe method to obtain the desired results (Figure 3.2)

It was imperative to determine whether the optimized technique could be performed by any trained technician and on any type of maize seed. The reason for this is that seed comes in various sizes and shapes (medium flat, medium round, etc). All sample groups

were analysed in triplicate in order to compare germination levels. Table 3.1 shows the level of germination on 4 lines and 2 hybrids. From these results, one could safely assume that this technique was consistently repeatable. With the exclusion of all the controlled parameters, error or non-germination could only be ascribed to human error. Control rows of seed were exposed to the same imbibing temperature and time were also incorporated to determine whether poor germination was a result of human error or just poor germination of that specific batch of seed. In every instance, the germination rates of the control rows matched those of the seed analysed using the half – seed technique, with a variation of between 1 – 5%. It was thus proven that the technique was reproducible and could be used as part of the total protocol.

Upon optimization, the technique proved to be efficient and reasonably straightforward for anyone to perform. This conclusion was derived from the fact that other technicians in the laboratory performed the same half – seed protocol and had similar germination results as that observed during the project. Germination rates between individuals did not vary that much, which indicated the ease of the technique. What one could conclude from this is that the technique was successful. One could successfully remove the fraction of seed required for analysis and germinate the rest of the embryo. Storage proteins and DNA could successfully be isolated from the same seed fraction. IEF and isozyme analysis on the extracted storage protein could then be performed. Real – time PCR was done on the extracted DNA and ELISA was performed on the proteins extracted from the leaves of the germinated seedlings. With all this information, the breeder would be able

to make successful selections from the samples that he submitted and he was able to make a direct comparison of each result to each plant.

The weight of the portion of seed removed depended on seed size and varied between 20 mg to 100 mg. The aim of the optimization of the half – seed extraction protocol was to not only to determine how much of buffer was required to obtain sufficient protein for IEF analysis, but also to choose an appropriate buffer that would allow us to potentially extract the Bt endotoxin. The reason for this was to allow us to analyse lines and hybrids for the GM trait as well as for genetic purity, simultaneously. Genetic purity is important for the breeder as it gives him a picture of the homogeneity of his line or hybrid. One is able to determine this by comparing the electrophoretic banding patterns from sample to sample. Although we could not distinguish Bt from non Bt samples, one still had information that was beneficial. The buffer selections were made based on the characteristic traits of the Bt endotoxin. The endotoxin was described to be active in very basic pH conditions and was 130 kDa in size. With reference to Table 3.2, 5 buffers were used with a pH range from 8.0 to 10. In addition, various volumes of buffer were added to the seed portion to determine which volume of extraction proved to give the most intense electrophoretic pattern.

Horizontal PAG electrophoresis allows one to focus on certain pI areas by the addition of ampholytes to the specific gel made. The gels used in the optimization, were of such a nature as to enable one to focus on a pI range in the area of interest that is specific to the proteins being analysed. In this study, the proteins of interest were described to be at a pH

range of approximately 9.6. This information guided the study to focus on buffers that could extract proteins with a basic pH nature, as well as optimize the IEF run on PAG gels with ampholytes that are conducive to isolating proteins in that pH region. Isoelectric focusing on ultra thin layer acrylamide gels is a powerful analytical tool which produces sharp pI ranges, with very narrow protein zones. Because of the high resolution protein separation, it results in more than 30 sharply separated bands after total protein staining (van Oers and Tamboer, 2005). Gels were also used that allowed one to look at specific isozymes which is more sensitive than total protein gel for variation between Bt and non Bt samples. Isozymes enable one to focus on a specific group of proteins in more depth. Isozyme analysis was performed with the hope of identifying a locus in a specific group of proteins that showed variation (Fig 3.6 and 3.7) that could be investigated for a possible link to the expression of the Bt endotoxin. There are a number of isozymes that could be employed, however the ones used in this dissertation (Fig 3.6 and 3.7) were selected based on the PAG gels used in this project. The isozymes selected were a: alcohol dehydrogenase (Fig 3.6), esterase (Fig 3.6) and malic dehydrogenase (Fig 3.7). None of these isozymes showed differences between the Bt and non Bt lines. However, esterase did show some bands that were not common to all the samples. This could have been a result of a mutation present in that specific plant or it could have been an off – type. An off – type is a plant that was pollinated with foreign pollen (not from the desired male plant) that was either introduced by an insect or by the wind.

There are various methods available for staining of proteins and three of these were investigated to determine their level of sensitivity, based on the concentration of proteins

present. Proteins were stained with coomassie brilliant blue for total protein staining, silver stain for total protein staining and isozyme stains (ADH, EST, MDH). Coomassie provides a simple and inexpensive way of looking at the storage proteins of maize. However, it is not as sensitive as the silver stain that can pick up protein bands that are present in lower concentrations. The isozyme stains are more specific than both coomassie and silver stain but are not as sensitive (Fig 3.6 and 3.7). With isozyme analysis one can only score a few loci for variation and as a result more than one type of isozyme was investigated to look for differences between Bt and non Bt lines and hybrids. Even at the isozyme level it is possible for one to pick up differences between lines or hybrids within the maize genus as seen in the figure below (van Oers and Tamboer, 2006).

Figure 4.1. Protein patterns on alcohol dehydrogenase enzyme stain analysed by horizontal IEF depicting differences between maize varieties.

The use of enzyme – systems for variety verification i.e., in kernels, limits the possibility to distinguish even a small amount of varieties (van Oers and Tamboer, 2006). Based on the ability to pick up differences on an isozyme level as described in Fig 4.1, the hypothesis was that if the Bt trait was introduced into one of the lines of a specific hybrid, and there were differences between those lines, the difference could be further investigated for a potential link to the Bt trait. However there were no differences

between the lines tested. This was expected as the chances of the Bt gene insertion affecting one of the enzymes selected were extremely remote.

All samples analysed on horizontal PAG IEF were run in triplicate upon optimization. Proteins denature rapidly at warm temperatures therefore precautions were observed to ensure that the expression of the proteins had were always consistent. Proteins are labile and as molecules go, proteins are relatively large and delicate and their shape is easily changed, (a process called denaturation) which leads to loss of their biological activity. This means that only mild procedures can be used and techniques such as boiling and distillation, which are commonly used in organic chemistry, are thus verboten (Dennison, 1999). The measures looked at included: the time of grinding, as prolonged exposure to grinding resulted in the denaturation of the storage proteins due to heat generated by friction, and the effect of proteins being frozen prior to it being analysed. Extraction of protein involved the grinding of the actual fraction with a metal grinder in the optimized extraction buffer. Proteins were extracted successfully each time in a ratio of 1:1 w/v. Samples run on one gel showed the same levels of polymorphisms to that of another gel of the same type. Staining of the gels with coomassie proved sufficient when compared to that of silver staining. The detection of the Bt trait on IEF was unsuccessful. The reason for this was probably the low concentration of the endotoxin present in seeds. Subsequent protein extractions on leaves were performed. Protein extracts from leaves from Bt and non Bt lines were also run on horizontal PAG electrophoresis. The reason for this was that the concentration of the Bt endotoxin is higher in leaf level than that of seed. AGBIOS (2004) describes the levels of the protein as being the highest in leaf tissue with

9.35 ug protein/g fresh weight as compared to 0.31 ug protein/g in grain. However, it was also impossible to isolate and identify the Bt endotoxin from leaves. This could be ascribed to a few factors namely: the extraction buffers used were unsuitable, the protein was not stable at the conditions they were subjected to, and the gel types and gel analysis were not sensitive to detect this protein. The Bt endotoxin, however, has been isolated previously on SDS PAGE (Fig. 4.2). However, the toxin was isolated from bacterial strains as opposed to plant material.

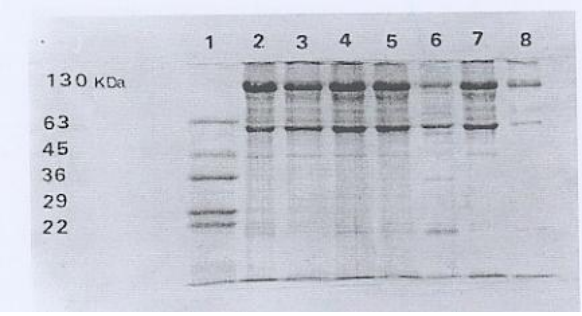


Figure 4.2. Protein patterns of *Bacillus thuringiensis* crystal analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (acrylamide, 13%) and coomassie blue staining. Numbers to the left indicate protein size in kDa. 1 = molecular weight marker, 2 – 8 = strain of *B. thuringiensis* Bands with molecular weights 130 kDa and 65 kDa represents the Cry1 and Cry2 proteins (Dias, *et al*., 1999).

SDS PAGE however is much more time consuming and tedious compared to horizontal IEF. With IEF it is possible to analyse up to 200 individual kernels on a single gel (Proteios, 2001) as opposed to only a few (5 – 20) on SDS PAGE. IEF is also faster than SDS PAGE. If the Bt trait was detected on IEF, it would have enable one to eliminate

traditional ELISA analysis and have the benefit of screening two tests at once (Bt trait and genetic purity). This would have resulted in a huge reduction in cost of sample analysis as well as time of analysis. Further investigation with regards to the isolation of this protein will continue. Detection of the Bt protein can be achieved by using a more sensitive total protein stain (e.g., Flamingo pink from BIORAD). Another avenue is by obtaining pure Bt endotoxin with known concentrations, and seeing if the protein cannot be detected on IEF. Attempts at developing an immunostain that is based on the principle of ELISA are also being investigated.

The results obtained from the electrophoresis still enabled us to determine variation between different lines and hybrids, as well as determine the genetic purity between kernels in a specific sample group (Fig 3.8). This allowed us to discard or remove the plants germinated that were shown to be impure or be genetically different to that of the other samples in the same sample group. As a result, IEF could be included as part of the laboratory protocol. Table 3.3 shows a summary of the electrophoretic results and all the samples showed a uniform banding pattern. Even though off – types might appear, there are intra laboratory standards that are set to determine whether a sample has passed or failed, depending on whether it is a line or a hybrid.

Table 3.4 depicts a summary of the samples that were analysed by Real – time PCR. The main aim of this analysis was to identify plants that were homozygous and heterozygous for the Bt trait. After running the samples on the instrument, the data was normally analysed using the ABI PRISM 7000 Sequence Detection System software to produce Ct

values of each reporter dye for each sample run. The amount of GMO material (FAM) dye is normalized to the amount of plant material (VIC) dye detected in each sample. This produces a delta Ct value which is averaged for replicate samples. These values are compared to a calibration curve produced from the delta Ct values of the known GMO concentration standards. This enables a %GMO result for each unknown sample to be produced (ABI PRISM Sequence Detection System, 2001). For the purposes of this study, each sample was run with an endogenous control. This control allowed one to determine whether the PCR reaction worked if the Bt gene did not amplify. This is because the test is based on a dominant Bt gene amplification (i.e. present or not). Of the 18 repetitions analysed, only two repetitions, showed to have 1 heterozygous plant in each of the repetitions. This enabled one to physically remove the heterozygous plants to ensure that the seedlings that were returned to the breeder for his breeding programme were all homozygous. From the results one can also successfully conclude that the real – time PCR technique was reproducible and reliable. This was expected as the protocol comes in a kit form and the PCR set up is a standard mix that is supplied by ABI. It is important for the breeder to know which plants are heterozygous for the trait because heterozygous plants have one copy of the gene and thus will segregate.

ELISA is a technique that has been used extensively in the detection of certain GM traits such roundup ready on maize and more specifically Bt on maize. Kits for the various traits are commercially available and are straightforward to use. ELISA is a simple, reliable and reproducible method for the detection of Bt in maize. Table 3.4 shows the results of the four lines and two hybrids analysed using the Envirologix Kit. All the

sample groups that had the Bt trait tested positive and the ones without the trait tested negative. The Enviroligix ELISA kit is expensive especially when large populations are screened. However, in comparison to real – time PCR it is much cheaper and can therefore be used as an less expensive prescreen for large sample groups that are tested for homozygosity of a line or a hybrid. This allows one to select all the samples that tested positive for the Bt trait and screen those only on real – time PCR as opposed to screening both positive and negative sample for Bt on real – time PCR. ELISA could not distinguish between homozygous and heterozygous plants. It would be interesting to determine whether quantitative ELISA (using a spectrophotometric plate reader) could detect differences in levels of gene expression and whether this could be correlated to homozygosity or heterozygosity. Due to the fact that the ELISA protocol is a kit, makes it highly reproducible. All samples were analysed in triplicate. Samples were run according to the instructions in the kit. There was a 100% correlation of results obtained between each set of ten samples (Table 3.5) each time the analysis was performed.

Over the last few years, it has been shown that molecular methods used in plant breeding have really improved the quality of breeding and also the rate at which new hybrids could be introduced into the market. What used to take years to achieve with traditional plant breeding, can now be achieved in a shorter period. With the use of protein and DNA technology, one can easily establish genetic purity and various traits in plants which could only be visualized previously after grow–outs has been done. With the introduction of GM crops, another avenue of competition in the seed market has been opened. In order

for breeders to stay competitive, it becomes vital that they are equipped with the necessary tools to aid them in their breeding programs.

The objectives of this study were not to only compare existing methods of testing for BT but also to optimize a laboratory protocol that could be used directly by the breeders to enhance their breeding programs. These objectives were fulfilled, in that the half – seed technique is now being used in our laboratory and over 2000 kernels have been tested using this method. The method proved to be economical and extremely successful. Breeders are confident that the plants that they are working with are exactly the ones that will have the quality traits they desire and they do not have to work with such large populations as they have been prescreened at an early age. Extraction of the protein and DNA for analysis has also been optimized and IEF results are consistent and full correlation has always been obtained. Although the BT protein could not be extracted for analysis on IEF, the IEF results are still useful in determining genetic purity and homogeneity of lines and hybrids. ELISA proved to be an effective and affordable prescreening technique to determine the presence/absence of the GM trait. RT – PCR is a quick, highly reproducible and straightforward method for determining homozygosity of plants. The only drawback is that it is expensive.

This study will enable breeders to develop future breeding programs that can incorporate any of the above – mentioned techniques to enhance their breeding programs. Breeding programmes require lines that are of the highest purity. Line purification of segregating lines can now be achieved by employing the half-seed protocol to isolate the various

differences and group those plants that are the same based on their genetic purity. Breeders can then plant those plants that have been grouped and determine which group will be suitable to achieve the desire hybrid. In the event of the line used being a transgenic line, one can add ELISA and/or PCR to the analysis and the breeder will then be ensured of having a homozygous, homogene group of plants. By using these techniques, a considerable amount of time is saved in terms of the breeding programme. A common factor of all these techniques is that they are not time consuming, especially when compared to traditional breeding techniques. Another advantage is that the techniques are not only limited to Bt testing but also crops with various other traits such as Round up ready herbicide resistance.

Another potential application resulting from this study is to use the half – seed technique and electrophoresis in Marker Assisted Backcrossing (MAB). In marker assisted backcrossing, the breeder aims to produce a line that has all the qualities of an elite line (recurrent plant) but with the addition of a specific trait from a donor plant. With the use an isozyme stain, one can distinguish between the recurrent and donor plant based on a specific marker. This enables one to select the desired off-spring by selecting the plants that shows identical banding patterns to that of the recurrent pattern. The advantage of this technology is that it is cost effective, reduces breeding time and ensures homozygous plants.

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