Assuring agricultural and food safety of genetically modified organisms (GMOs) in Southern Africa (GMASSURE) – Background information

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1.1. Introduction

The following literature review has been compiled to give an in-depth understanding into the possible unintended effects of transformation following the production of genetically enhanced plants. Since this study focusses on the investigation of possible transformation-induced unintended alterations to transgenic plant genomes, aspects that collectively constitute possible transformation and post-transformation events will be briefly described, as well as gene expression analysis as the main theme of unintended effects analysis. Historical and current safety assessments will also be briefly discussed. In order to dissect this topic, a foundational overview will be given on the model plant, which is potato, fungal pathogens, pathogenesis and fungal inhibitors, such as polygalacturonase inhibiting proteins (PGIPs). This will assist in understanding the overview on genetically modified (GM) plants, their production purpose, with special focus on transgenic plants produced for fungal disease resistance.

1.2. Genetically modified crops, food and feed

1.2.1. Background on GM crops, food and feed

GM crops have been developed over the years for improvement of desired traits for enhanced agricultural production, as well as to facilitate less use of agricultural pesticides (Brookes and Barfoot, 2013; Brookes and Barfoot, 2014). The technology employed to produce GM crops has been described as advantageous when compared to conventional plant breeding, since the desired traits can be obtained in a relatively shorter period. In addition, the technology is said to enable the introduction of desired characteristics that cannot be accomplished solely through conventional plant breeding.

By 2020, the population of Asia, Africa and Latin America will have increased drastically, leading to an increased number of people that rely on agricultural crops for food and feed and to generate an income (Visser *et al*., 2009). Research shows that in 2001, food consumption per person measured at kilocalories per day was 2789 (Walker *et al*., 2011; Kearney, 2010). However, and of great concern, projections show that by 2050, this will increase to an estimated 3130 kilocalories per person per day. This increase is owed to research findings that show that by 2050, the world population is expected to double to an estimated 10 billion (Plahuta and Raspoor, 2007; Hijmans, 2003; FAO, 2009). Factors that have been considered important to cope with the increasing food demand globally include, amongst others, the development of crop varieties with improved taste or convenience, development of varieties with improved nutrition and yield, development of varieties that require the use of less water and fertilizers, and the production of disease resistant varieties (Lu and Snow, 2005; Bennett

et al., 2005; Lee *et al*., 2006; Plahuta and Raspor, 2007; Zhao *et al*., 2007; Lu and Yang, 2009; Faulkner, 2011; Lusser *et al*., 2012).

Genetic modification, also referred to as breeding of new plants by recombinant DNA technology, is the alteration of genetic material that would not occur naturally (Bakshi, 2003; Heckmann *et al*., 2006; Ekici and Sancak, 2011). It is a technology that has been said to be both economically and nutritionally important during the improvement of the quality and quantity of food to meet the realised increasing food demands brought about by the increasing world population (Bennett *et al*., 2005; Plahuta and Raspor, 2007; Ekici and Sancak, 2011). This has been said to be a significant supporter of agricultural productivity (Bennett *et al*., 2005).

Traits that have been used to improve crops are traits such as herbicide and insect resistance, salt and drought tolerance, yield increase, high protein content and vitamin A enrichment (Ye *et al*., 2000; Toyama *et al*., 2003; Cao *et al*., 2004; Bahieldin *et al*., 2005; Huang *et al*., 2005; Tang *et al*., 2006; GMO-permits-2013). Pest-resistant and herbicide tolerant varieties were the first products of GM technologies and these saw commercialisation in the mid-1990s (James, 2010). Since then, an estimated 160 million hectares are being utilised by GM varieties in 29 countries worldwide (James, 2010).

Any GMO activities in South Africa require a permit that can be obtained from the Department of Agriculture, Forestry and Fishery (DAFF). (www.biosafety.org.za). There are six catergories under which permits are issues to applicant and these are for export, import, commodity clearance, general release, field trials as well as contained use. Several companies applied for GMO permits for either the exporting or importing of GM crops (GMO-permits, 2013). The GM crops include amongst others drought tolerant maize applied for supplying by Monsato, herbicide tolerant maize applied for supplying by Pannar and Pioneer, insect resistant and herbicide tolerant maize applied for supplying by Monsato and Pioneer, insect resistant maize applied for supplying by Monsato and Pioneer, insect resistant and herbicide tolerant cotton applied for supplying by Mosanto and Bayer and herbicide tolerant soybean applied for supplying by South Hemisphere (GMO-permits, 2013). The permits were requested by the companies for various reasons which include export for commodity, import for contained use, export for planting, export for contained use and import for planting.

1.2.2. Benefits and shortcomings of GM crops, food and feed

Benefits and shortcomings of GM crops, foods and their products have been documented since 1986 to date. The highlighted advantages of GM crops, food and feed include, amongst others, the ability of these to enhance food security, particularly to small-scale and resource-poor farmers in developing countries (Azadi and Ho, 2010; McGloughlin, 2010; Anthony and Ferroni, 2012). Some of the noted benefits include increased crop yield in a relatively shorter period, reduction in the utilisation and application of plant protection chemicals, production of crops with tolerance to environmental stresses, reduction in labour input, and production of foods that are affordable with enhanced nutritional content (Kuiper *et al*., 2003; Sharma, 2003; Azadi and Ho, 2010; Anthony and Ferroni, 2012). These benefits have been said to, overall, improve agricultural production and plant breeding in developing countries.

However, the documented benefits have been outweighed by shortcomings and concerns. Some of the issues brought forward as shortcomings of GM crops, foods and their products include, amongst other, potential toxicity of GM food and feed, and the assumption that the products may contain allergens, and the possible development of antibiotic resistance from the utilisation of GM crops, food and feed (Coleman, 1996; AgResearch, 2001; Malarkey, 2003). Genetically, concerns about GM crops, food and feedhighlight the possible unintended transfer of genes from GM to non-GM crops or to the wild type, the evolution of GM crops into becoming weeds, the direct and indirect impact of GM crops on non-target species, environmental risks associated with GM crops, and the impact of GM crops on genetic diversity (OECD, 1993; AgResearch, 2001; Dean and Shepherd, 2007; Bakshi, 2003). Directed at the small-scale and resource-poor farmers, the highlighted concerns also include production of food products with inferior quality and hindrances that may result in limited access to seeds created by patents on the GM crops (AgResearch, 2001).

Furthermore, contrary to the highlighted benefits of GM crops, crops with enhanced resistance have been viewed as having a one-sided benefit to commercial farmers that grow the crops and companies that own the production technologies (Bakshi, 2003; Ekici and Sancak, 2011).

1.2.3. General views on the safety of GM crops, food and feed

Some consumer concerns brought were forward regarding the safety of GM crops, food and feed (Kearney, 2010). As a result, the production of GM crops led to increased investigations within government regulatory boards in terms of research required to determine the safety of these products (Plahuta and Raspor, 2007). Activists, particularly in Europe, have intensified their opposition to the introduction and production of GM crops, food and feed (Hall, 2008;

Azadi and Ho, 2010; Dibden *et al*., 2013). To date, no international consensus has been reached for evaluation of the safety of genetically modified plants for consumption. Over the last decade, the safety of genetically modified crops in animal feed or food for human consumption has been routinely tested in some countries. Protein- and DNA-based methods have been developed for detection of genetically modified organisms. Worldwide, legislation now faces questions on the use and labeling requirements of genetically modified crops and their derivatives. Still, there are concerns about the safety of genetically modified crops (AgResearch, 2001; Mayet, 2013). Also, genetically modified crops could contain toxic substances or larger amounts of heavy metals and the crops might not be substantially equivalent in genome, proteome, and metabolome to traditional untransformed counterpart. The toxic substances may be produced in the GM crop as by-products of the expression of the transgene. An additional concern is that contrary to expectations, GM crops may be less nutritious due to disruption in the expression of endogenous genes that codes for proteins that produce beneficial foods for the consumption. This review will focus on perspectives of the genetically modified food crops.

It has been suggested that the focus to assist with food security in developing countries should be breeding for highly nutritious food crops that are diverse in terms of abiotic and biotic stress resistance and tolerance (Gaskell *et al*., 1999; Visser *et al*., 2009). The traits that are of importance for genetic improvement of crops include, amongst others, a broad-spectrum of disease resistance and tolerance, high yield in different climatic conditions, high storage quality, and appropriate applicability of the crop for processing and market consumption. However, one of the important factors that is always investigated by risk assessors and regulators when dealing with GM crops, is the breeding technique used to produce the crop and how the technique has been refined for adoption in commercial breeding programs (Schaart and Visser, 2009; Kuzma and Kokotovich, 2011; Tait and Barker, 2011). Plant breeding techniques have evolved over time and have seen migrations from the use of physically induced mutagenesis to improve traits, to the use of cell biology and tissue culture for micropropagation (ISAAA, 2010; McInnes and Voelcker, 2014; Moffat *et al*., 2014; Pasqual *et al*., 2014). The migration then saw movement to double-haploid techniques that enabled, amongst others, crossing between two incompatible plants, which was followed by the modern biotechnology era that was initiated by molecular marker-assisted selection (MAS) to select agriculturally important traits (ISAAA, 2010). Other developments following MAS, included recombinant DNA technology which afforded breeders a diverse gene pool for trait selection, targeted deletion or insertions of genes into genomes, and site-directed mutagenesis to modify gene functions (Zhu *et al*., 2000; Townsend, 2009; Kuzma and Kokotovich, 2011).

South Africa and Norway initiated an Environmental Biosafety Cooperation Project (EBCP) between 2008 and 2010 aimed at monitoring the safety of GM crops on the environment (www.environment.gov.za). Certain monitoring guides, prior release, were set and these include developing a monitoring plan to evaluate the impact of GM crop on the environment, evaluation of the impact on agricultural practices, monitor the development of resistance of insects and keeping records of the locations of GM planting.

1.2.4. Resistance towards GM crops

In March 2012, BASF announced intentions to terminate the breeding of GM crops specific to European markets, but instead focus resources on the American and Asian markets (Dixelius *et al*., 2012). This decision was founded on the constant restricting legislatures on GMOs that is within the EU. Furthermore, the decision were based on the volatility experienced from political and market mistrust that had translated into negative impacts on innovative research for European agricultural research. The departure of BASF from European GMO research is thought to have forthcoming negative effects on European agriculture, as well as food availability, particularly on potato production. The emphasis on the negative impact on potato production was solely based on some of the main research focus areas that were conducted by BASF on this crop. BASF had developed potato varieties with altered starch qualities for industrial starch production and bred a cultivar for enhanced resistance to *Phytophthora infestans* (van der Vossen *et al*., 2005; Laursen, 2012). One of the varieties with an altered starch quality, Amflora, was one of two GM crops that have been approved by the EU for cultivation.

The abandonment of these GM varieties by the EU was mostly informed by the financial calculations that were made when the release of these varieties was considered (Dixelius *et al*., 2012). These financial implications included, amongst others, the impact of the varieties on the producers' work force and capital, the costs that will be incurred in separating the GM crops from the non-GM, as well as in determining the then yet unknown environmental impact of the GM crops. The value attached to these financial implications was set at $16 million annually (Dixelius *et al*., 2012). The penalties, however, for the abandonment that led to the withdrawal of BASF, include amongst others, the forseen increased dependence by european farmers on environmentally unfriendly fungicides as control for *P. infestans*, an increase in disease pressure and a potential decrease leading to depletion of researchers, since research units will not be able to sustain themselves, since career prospects in this field will be seen as less attractive with these hindrances. Furthermore, another penalty will be an increase in potato imports to satisfy the high potato consumption in Europe.

An additional reason for political resistance against GMOs stems from the fact that the majority of the crops that are currently commercially available are under heavy patents by seed companies, such as Monsato and Pioneer Hi-Bred (Grushkin, 2012). However, research shows that by 2014, a vast majority of earlier patents on GM crops will expire. These include GM crops such as Roundup Ready soy seed which occupies the majority of GM land. In South Africa, cultivars that are being planted are AG 5601, A 5509 RG and AG 6101 (Monsanto-SA, 2004). These cultivars were found to occupy an estimated 500, 000 ha in 2012 in South Africa. This expiration will serve as an opening for other companies to produce other varieties that may compete within the market, thus challenging the already known GM varieties. In addition, it will elicit innovative competition in terms of traits to be investigated which were previously not considered.

In South Africa, resistance towards GM crops, food and feed has been voiced by numerous groups, which include the Landless People's Movement, the South African Peace Network, Earthlife Africa and Food and Trees for Africa (www.acbio.org.za). An example of the ressitance was seen in a letter written to the CEO of Tiger Brands titled "African demand for Tiger Brands to go GM-Free" (Mayet, 2013). The letter states the lack of evidence of the long-term safety of GM crops, food and feed on human, animal and environmental health. This lack of evidence was highlighted, in the letter, to be the causal agent for the banning of GM crops, food and feed in over 40 countries in the world. The argument in the letter further presented crucial findings on the presence of GM on the food consumed daily by South Africans, produced by Tiger Brands. These findings include Ace super maize meal contains 78% GM maize, Ace instant porridge contains 68% GM maize, Ace maize rice contains 70% GM maize, Jungle B'fast energy cereal contains 41% GM maize and Lion samp and beans contains 48% GM maize (Mayet, 2013. www.acbio.org.za).

Extensive safety assessments are an important component of the production of GM crops (Shepherd *et al*., 2006; Wilson *et al*., 2006). There are factors that are important to interrogate during the production of transgenic crops and these stem from the fact that the production of these GM crops is as a result of genetic manipulation, thus it is important to understand the impact of the expression of the "foreign" transgene on the expression of endogenous genes and on the host plant as a whole (Cellini *et al*., 2004; König *et al*., 2004).

The South African regulatory framework stipulates that it is important to ensure that the GM crop and activities performed with the crop and its products are assessed with regards to any potential risk they may have on human, animal and environmental health (www.daff.gov.za). South Africa forms part of the Organisation of Economic Cooperation and Development

(OECD), the Food and Agriculture Organisation of the United Nations (FAO), the World Health Organisation (WHO) and the International Life Science Institute (ILSI) thus documents developed within these bodies have contributions from a South African perspective. Guides, codes of practices, standards as well as recommendations on food safety are set by the Codex Alimentarius Commission. In South Africa, these guides are implemented to promote responsible development of the safe use of GM crops, food and feed as stipulated in the GMO Act of 1997.

1.3. Techniques used for the production GM crops

1.3.1 Traditional molecular plant breeding

Plant breeding has also been termed applied genetics because it uses a combination of natural genetic variation and artificial selection, and induces new variability in an artificial process (Allard, 1999). The genetic variation has been artificially manipulated through controlled plant breeding. This practice has greatly influenced breeding practices on the genetic composition of plant crops.

Molecular plant breeding deals with the integration of exogenous DNA into the plant genome using gene transfer technologies (Hansen and Wright, 1999). Two methods are commonly used for exogenous DNA transfer into plants, and these are particle bombardment and *Agrobacterium*-mediated transformation (De Block, 1988; Birch, 1997; Harwood *et al*., 2002). The particle bombardment transformation method, also known as the biolistic transformation, is based on the use of acceleration to physically deliver DNA-coated gold or tungsten micro-projectiles into the target plant genome and is commonly used to transform plants that are not susceptible to *Agrobacterium* transformation (Christou, 1995). On the other hand, *Agrobacterium*-mediated transformation utilises the ability of *Agrobacterium* to copy and transfer a specific portion of DNA (T-DNA) present on a tumour-inducing (Ti) plasmid into the nucleus of the plant cell to allow for the integration of the DNA into chromosomes and subsequently leading to the integration of the T-DNA into the plant genome.

The most common method of transformation used in potato has been found to be *Agrobacterium tumefaciens* transformation (Romano *et al*., 2001; Veale *et al*., 2012). However, there are species have been identified that cannot be efficiently transformed by *Agrobacterium* (Figuera Filho *et al*., 1994). Such species have been ideal for transformation using particle bombardment (Romano *et al*., 2001). *Agrobacterium*-mediated transformation has been found to be limited to the integration of a maximum of three genes on a single plant

transformation construct, with at least one of the genes being a selectable marker (Romano *et al*., 2001).

The integration of transgenes into a host plant genome, following particle bombardment, has been said to non-randomly occur at AT-rich regions carrying the matrix attachment region (MAR) motifs (Morikawa *et al*., 2002). MARs have been defined as nuclear matrix prone DNA elements only found in eukaryotic genomes (Bode *et al*., 2000).These elements have been postulated to be target sites for transgene integration into the host plant genome (Morikawa *et al*., 2002). Their function has been explained as creating open chromatin to make the host plant genome accessible to transgenes.

In potato, transgenes have found to integrate into the plant's internodes, leaves and tubers when both the *Agrobacterium*-mediated transformation and micro-projectile techniques are used (De Block, 1988; Romano *et al*., 2001). An advantage described for *Agrobacterium* transformation was the ability the technique has to allow for the integration two T-DNAs into the host plant genome (De Block and Debrower, 1991; De Buck *et al*. 1998). Since a T-DNA comprises the transgene expression cassette as well as the *in planta* expression promoter, the integration of two T-DNA translates to the integration of two transgenes into a single host genome. On the other hand, reports have shown that particle bombardment can also be used to transfer two T-DNAs into the host plant genome (Hilliou *et al*., 1999).

Eukaryotes have two major plant genetic recombination mechanisms, and these are homologous recombination (HR) and illegitimate recombination, which is a form of the non-homologous end-joining (NHEJ) process (Schnable *et al*., 1998; Haber, 2000). The predominant form of recombination found in plants is the NHEJ process thus described as illegitimate recombination.

The experimental design of *Agrobacterium*-mediated transformation has been divided into three stages, namely, initiation, bacterium-to-plant transfer and nucleus targeting (Gelvin, 1998; Ward and Zambryski, 2001; Gelvin, 2003). The intiation stage entails the idenfication and isolation of the gene of interest followed by the insertion of the gene into a suitable functional construct. The construct includes the gene expression promoter, gene of interest, selectable marker as well as codon modification. The initation stage then continues to the insertion of the transgene into the Ti-plasmid. The final step of the initiation stage involves the insertion of the T-DNA, which contains the transgene, into *Agrobacterium*. The next stage of Agrobacterium-mediated transformation is the bacterium-to-plant transfer. During this stage, the transformed *Agrobacterium* is mixed with plant cells to facilitate the transfer of T-DNA into

the plant genome. The final stage of *Agrobacterium*-mediated trasnfromation is nucleus targeting where the transgene is randomly integrated into the plant chromosome. Following nucleus targeting, NHEJ enables the integration of T-DNA into the plant genome in the absence of any homology between the T-DNA and plant DNA sequences (Mayerhofer *et al*., 1991; Ohba *et al.*, 1995).

1.3.2. Newer plant breeding techniques

The innovation, introduction and implementation of newer plant breeding techniques gave birth to new regulatory frameworks in the areas within which the techniques were being introduced. The frameworks encompass legal classification, regulation and governance of the GM techniques, and the release of GM crops into the environment (Schaart and Visser, 2009; Kuzma and Kokotovich, 2011; Tait and Barker, 2011).

Several new plant breeding techniques have been investigated to understand the current status of research and development, in terms of the applicability of the techniques, the viability of these techniques as tools for commercial breeding, constraints experienced with the utilization and implementation of the techniques, and crops produced using the selected techniques (Lusser *et al*., 2012). The techniques that were selected and analysed through the European Union's Joint Research Centre (JRC) included agro-infiltration, cisgenesis and intragenesis, grafting on to GM rootstock, oligonucleotide-directed mutagenesis (ODM), reverse breeding, RNA-dependent DNA methylation (RdDM), and zinc-finger nuclease (ZFN) technology (Zhu *et al*., 2000; Aufsatz *et al*., 2002; Schouten and Jacobsen, 2008; Stegemann and Bock, 2009; Dirks *et al*., 2009; Vezina, 2009). The popularity of these techniques were based on available publications on the techniques, and it was found that most work was done using cisgenesis and intragenesis, followed by RdDM and grafting on to GM rootstock, then agro-infiltration and ODM, which were followed by ZFN technology, with few publications found on reverse breeding, as it was a newer technique (Lusser *et al*., 2012).

To better understand the applicability of the investigated techniques, the plant species used, as well as traits introduced, were examined (Lusser *et al*., 2012). Findings revealed that the ODM technique can be used on a variety of crop plants, which include banana, canola, maize and wheat, and ZFN technology was restricted to maize and soybean. The RdDM technique has also only been able to be applied to a limited number of plants and these are carrot, maize and potato. RdDM was used to breed for modified starch content in potato. Since cisgenesis, intragenesis and grafting on rootstock depend on genetic transformation, a wider range of crop plants were successfully explored using these techniques. Cisgenesic and intragenic

approaches were used to introduce black spot bruise tolerance, fungal resistance and lower acrylamide levels in potato (Holme *et al*., 2013). Grafting facilitated breeding for potatoes that are resistant to fungi and viruses. Important agricultural crops were successfully screened using agro-infiltration and these included crops such as beans, rice, potato and tomato. In potato, agro-infiltration was used to screen for resistance against fungi and viruses. Since reverse breeding is a newer technique, no conclusive data is currently available. The number of patents per technique were also analysed to determine the commercial exploitation of the technique which translates to the technique's potential application (Lusser *et al*., 2012). The ODM technique was found to have the most number of patents followed by cisgenesis and intragenesis. These were followed by ZFN and grafting on GM rootstock. Agro-infiltration followed grafting in patent rankings, with reverse breeding and RdDM having the least number of patents, at two and one, respectively. Private US institutions held 65% of the analysed patents and 26% were assigned to the EU.

The results obtained from patents, crop plants applicable for the techniques, as well as amount of research conducted using the techniques provided insight into the potential of the use of each technique by commercial breeders (Lusser *et al*., 2012). A survey of public and private companies was conducted to determine the applicability of these techniques for commercial breeding and analysis of progress made in commercial development. The four phases of commercial development are phase 1, which means the construct is being optimised and is being used in target crop, phase 2 is a stage where the trait has been developed with pre-regulatory data available and large-scale transformation is in process. Phase 3 is the commercial development stage at which the trait has been integrated into the target crop and field testing is underway. In addition, the necessary regulatory data is being compiled at this stage. Lastly, the commercial development of a crop enters phase 4 where the necessary regulatory submissions are made and seeds are produced for pre-marketing. Oilseed rape and maize are two products obtained from the ODM technique with tolerance to herbicides and were both found to be at commercial development phases 2 and 3. Agro-infiltration has been used to breed for lettuce with resistance to downy mildew, and oilseed rape and potato with undisclosed traits. The products' advancement was not disclosed. Maize, oilseed rape and potato varieties were produced to contain undisclosed traits and results showed that the process was at commercial development phases 1 to 3. The products of the RdDM technique were found to be at commercial development phase 3, and these are maize and oilseed rape with the traits of interest not disclosed. Maize, tomato and oilseed rape varieties were produced using the ZFN technique with undisclosed improved traits, but were however said to be at commercial development phase 3. Products from reverse breeding were found to only

be at the research phase. No results in the advanced commercial phase were found for products of grafting on GM rootstocks.

The advantages of the use of these techniques for crop improvement include factors such as the relatively lesser amount of time required to obtain the final product when compared to conventional breeding (Li *et al*., 2007; Lusser *et al*., 2012). This can also be seen with an earlier generation of returns from the market. As soon as the final product has been obtained and it is termed a GMO, several factors come into play and these come in the form of time and financial implications of the research related to regulations of GMOs (McElroy, 2003; Bradford *et al*., 2005; Kalaitzandonakes *et al*., 2007). Research shows that an estimated $35 million may be needed per GM event to generate the required regulatory data, with a waiting period of 5.5 years (McDougall, 2011). The extended time, coupled with high cost, has shown to be a potential deterrent for the use of the new breeding techniques that lead to GMO production (Kalaitzandonakes *et al*., 2007; Miller and Bradford, 2010).

1.4. Safety assessment of GM crops, food and feed

With the realised increase in the adoption of GM technology and products as part of food insecurity mitigation, environmental and human health risks cannot be ignored when the importance of GM crops is being investigated (Emiroglu, 2002; Bakshi, 2003). The current techniques utilized for the safety assessment of derivatives of genetic modification, particularly crops, food and feed-based derivatives, evolved from collaborations between international agencies, which include the United Nations World Health Organisation/ Food and Agricultural Organisation (FAO/WHO, 1991, 2000) and the Organisation for Economic Co-ordination and Development (OECD, 1993). The techniques entail a comparative assessment between the characteristics of the modified crop/ food/ feed and an existing crop/ food/ feed which is usually the parent crop from which the genetic modification was developed.

Research was conducted in 2003 and 2004 by the International Council for Science (ICSU) and the United Nations Food and Agriculture Organization (FAO), and results showed no evidence of adverse effects of GM crops on the environment as well as no toxicity presented by the consumption of foods derived from GM crops (ICSU, 2003; FAO, 2004). The results further showed that gene transfer that occurred from GM crops to the wild-type relative was similar to the occurrence obtained from traditional crops. Further research conducted on the environmental impact showed no evidence of negative effects on the environment by GM crops (Sanvido *et al*., 2007).

Concerns that have been raised in terms of the safety of GM crops, environmental risks, protection of biodiversity and impact on human and animal health have been investigated through the Cartagena Protocol on Biosafety of which South Africa is a signatory (Secretariat of the Convention on Biological Diversity 2000) (Kearney, 2010). This protocol has been used by countries to develop national GMO regulatory frameworks. In South Africa, a Genetically Modified Organisms Act was revised in 1997 as an adaptation of the Cartagena Protocol on Biosafety (Act No. 15 of 1997) (www.daff.org.za). Details required for application of the release of GMOs include a description of the GM plant, the GM trait, as well as the country of origin of the GM plant. Furthermore, requirements include general information on the release of the GM plant, description of GM-derived products and its use, and description of field trials undertaken for the GM plant. In addition, details required for the release of the GM plant include description of the pollen spreading characteristics of the GM plant, handling of seeds and the vegetative reproduction methods of the plant. Moreover, information is required on transgenes and their respective products which include information such as transgene expression levels, declaration on whether the expression is constitutive or induced and expression site on the plant. Additionally, information on the potential resistance to environmental or biological conditions, potential risks to human and animal health according to the Occupational Health and Safety Act of 1993 (Act No. 181 of 1993), potential long-term impact of the GM plant on biotic and abiotic components of the environment, and socio-economic impact of the GM plant on communities in the proposed release region. The release also requires information on how the GM plant will be monitored, how possible pathogenic and ecologically disruptive impacts will be evaluated, how unused parts of the GM plant will be disposed of and measures that will be used for risk management.

The European Commission (1997) identified three possible outcomes following safety assessment studies. Firstly, the modified food can be homologous to the traditional food or ingredient, thus eliminating the need for further testing. Secondly, the modified food can be homologous to the traditional food, with some distinctly characterised differences, in which case safety assessments targeted at the differences must be performed. Thirdly, the modified food can stand apart from the traditional counterpart in numerous and complicated aspects, or no traditional counterpart is available. In this instance, the modified food will require a comprehensive assessment similar to that discussed by König *et al*. (2004). This may be due to the fact that the endogenous genes and their functions will possibly be disrupted through the random integration of the transgene in the plant DNA. These effects of transformation are termed ‘unintended’ or ‘non-target’ effects as they occur secondary with the the primary aim being is crop improvement (Müller *et al*., 2006; Shepherd *et al*., 2006; Wilson *et al*., 2006).

Several authors have highlighted that techniques utilised for targeted analysis of the comparative study between the GM crop and its traditional counterparts are biased (Cellini *et al*., 2004; Wilson *et al*., 2006). The criticism emanates from the fact that the use of these techniques do not allow for the analysis of possible unintended effects that could directly or indirectly arise from the genetic modification. To eliminate parts of the biasness of comparative studies between the GM and traditional plants, the plants have to be grown under the same regimes and environments.

Prior to studying the possible unintended effects of recombinant DNA techniques, it is important to understand the definitions of these effects. There are intended effects of genetic engineering and these are changes that occur following genetic modifications which are aimed to take place as a result of the introduction of the transgene and will consequently result in the accomplishment of the original objective of the genetic engineering process (Cellini *et al*., 2004). Unintended effects are those changes that occur following genetic engineering where significant differences are found in the response, phenotype and composition of the GM plant when compared with the traditional plant from which it is derived.

Unintended effects have further been divided into ‘predicable’ and ‘unpredictable’ unintended effects (Cellini *et al*., 2004). Predictable unintended effects are changes that exceed the primary expected effects of the introduction of the transgene, but are, however, applicable through the aid of the current knowledge of plant biology and metabolic pathway integration. On the other hand, unpredictable unintended effects are changes that are currently undefined and not clearly understood. Methods that can be exploited to determine the presence of unintended outcomes of transformation include, amongst others, determining the transgene integration site/s, the events that occur during the integration of the transgene into the host plant, as well as gene expression analysis of the transgenic genome compared to the traditional counterpart, thus showing the impact of transformation on the expression of endogenous genes.

1.4.1. Molecular characterisation of GM crops, food and feed

Guidelines have been set for the molecular characterization of GM crops prior to market and commercial release and these were placed under six categories (Wetenschappelijk Instituut Volksgezondheid, 2003). These categories are (i) description of the genetic material used for the transformation, (ii) description of the transformation method, (iii) description of the transgene loci, (iv) transcript and protein characterization, (v) inheritance and stability of the transgene and (vi) detection and identification of the transgene. The requirements set out for

the (i) description of the genetic material used for the transformation include information on the plasmid used in the production of the transformation recombinant, detailing genetic elements such as the orientation and position of the transgene expression cassette within the vector, (ii) the restriction endonuclease sites of the transformation construct, (iii) clearly marked T-DNA borders and promoters, (iv) origin of the donor organism, and (v) a description of the pathogenicity of the expressed protein, and (vi) information on the biochemical, molecular and physiological properties of the transgene product.

The requirements under description of the transformation method include details on the transformation protocol used to obtain the transgenic plant, which also includes the bacterial strain used if *Agrobacterium*-mediated transformation protocols were used (Wetenschappelijk Instituut Volksgezondheid, 2003). The guideline also stipulates that the transgene loci be described before the GM crop is released to the market. In order to comply, the number of insertion events of the transgene must also be supplied, as well as the transgene insertion site/s. Insertion site detection is expected to be presented as the transgene sequence accompanied by approximately 500bp of plant DNA in both flanking regions. Thus, a detailed description of the flanking sequences should also be included in addition to the transgene sequence. Novel chimeric open reading frames (ORF) should be described and their functionality evaluated. If the flanking sequence contains part of the chimeric ORF, it is expected that more sequencing must be performed beyond the 500bp radius until a putative ORF is obtained.

A detailed characterization of the transcript and the encoded protein is required as part of the guidelines for safety assessments of GM crops before commercial release (Wetenschappelijk Instituut Volksgezondheid, 2003). A detailed analysis of the expression of all detected ORFs should be provided together with methods used to extrapolate the expression. Expression entails, amongst others, details on the tissue specificity of the transcript and protein expression, as well as levels of expression, and translation of the transcript to protein. Furthermore, a detailed narrative of the expressed protein/s should be provided, as well as the stability of the protein/s in the cell and in the surrounding environment. Moreover, the guide states that a detailed narrative should be provided on the transgene and expressed proteins in terms of inheritance. In addition, evidence should be provided to show that the transgene trait is maintained and expressed during propagation of vegetatively reproducing crops. Additionally, the guide dictates that the sequences of the oligonucleotides that were used to isolate the transgene should be provided together with reference transgenic and control material.

The main objective of the safety assessment is to demonstrate that genetically modified food and feed are equivalent to their traditional counterparts, where one exists, and that there are no introductions of any additional or new risks to consumer health (Cellini *et al*., 2004). These assessments are put in place to quantitatively detect or identify the GM crops, food and feed that are being introduced into the market (Stein and Rodriguez-Cerezo, 2010).

1.5. Aspects of possible unintended effects in transgenics

1.5.1. Integration of transgenes into plant genomes

Pawlowski and Somers (1996) demonstrated that single copy or repeated and multiple insertions of exogenous DNA can occur during genetic engineering. Laufs *et al*. (1999) and Koncz *et al*. (1989) further showed that multiple insertions can take place into linked or unlinked sites. Moreover, Fladung (1999), and Kumar and Fladung (2000) demonstrated that following transformation, the transgene may be unstable within the host genome, and the insertion site may also be unstable owing to the transgene instability.

Directed and inverted repeats are some of the complex integration patterns which have been found to result from *Agrobacterium*-mediated transformation (Kritzkova and Hrouda, 1998). Inversion (Laufs *et al*., 1999) and translocations (Castle *et al*., 1993) have been found to be some of the types of chromosomal rearrangements linked to T-DNA insertion occurring at the insertion site in the plant genome. Vector-based filler DNA has also been observed following the integration of exogenous DNA into the plant genome. Vector-based filler DNA has been characterised as non-T-DNA sequence from the transformation vector backbone. Plant based filler DNA has been found between T-DNA repeats (Kumar and Fladung, 2000; Filipenko *et al*., 2007), whereas vector-based filler DNA sequences were found outside the left and right borders of the T-DNA (De Buck *et al*., 2000). The plant based filler DNA is said to be an important facilitator of the integration of T-DNA into plant chromosomes (Kumar and Fladung, 2000). However, some filler DNA cannot be characterised since they fragment sizes are too small and range between 1 to 22 bp in length (Kim *et al*., 2003; Filipenko *et al*., 2007).

Kononov *et al*. (1997) found that *Agrobacterium*-based integration occasionally caused the recurrent integration of T-DNA vector backbone sequences into the transgenic plant genomes. It is possible to have vector backbone flanking the right border (RB) integrated into the host plant genome following transgene insertion (Lee *et al*., 2004). This event has been hypothesised to be the result of T-DNA processing that occurred where, instead of the insertion initiated at from the RB, this initiation site is skipped and T-DNA insertion occurs from the LB.

1.5.2. Transgene integration sites distribution

Predictions into the fate and integration site of a transgene into the plant genome are not possible due to the genome's nucleotide sequence (Cellini *et al*., 2004). Several authors have used various genetic mapping techniques to demonstrate that, in several plants species, transgenes integrate throughout the entire plant genome without any preference for a specific chromosome (Thomas *et al*., 1994; Jacobs *et al*., 1995). However, T-DNA containing transgenes have been found to show preference towards gene rich-regions (Koncz *et al*., 1992). This preference has been found to be responsible for disruptions to endogenous gene function. Barakat *et al*. (2000) also showed that T-DNA has preference towards gene-rich regions during a study performed on Arabidopsis and rice. It was found that there was a correlation between the different gene distribution and the pattern of integration of transgene in the two plant genome.

Several cytological methods have been employed to detect transgene chromosomal location and structure, and these include genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH) (Iglesias *et al*., 1997; Pedersen *et al*., 1997). These methods have assisted some researchers in identifying the transgene integration site/s at the subtelomeric and telomeric regions of individual chromosomes (Svitashev *et al*., 2000).

In addition to the cytological methods, identification of the transgene insertion site has been done through direct sequencing of flanking DNA following the rescue of clones carrying transgene/genomic DNA junctions (Thomas *et al*., 1994; Liu *et al*., 1995; Fladung, 1999). A high correlation was found between complex integration patterns and transgenic loci with unstable gene expression (Matzke and Matzke, 1998; Fladung, 1999; Laufs *et al*., 1999). As a result, it was concluded that the determining factors of the stability of an expressed gene are the identification of the precise site of transgene integration, as well as the structure of the integration site. In addition, it was found that the locus of transgene integration and the regions surrounding the site of insertion are crucial for the stable expression of a transgene (Iglesias *et al*., 1997; Gelvin, 1998).

Studies of transgenic tobacco indicated that chromosome telomeres are preferred by stable inserts where no binary vector sequence is present (Iglesias *et al*., 1997). On the other hand, the integration of transgenes was found to have preference for the distal part of chromosome arms which are gene-rich regions (Barakat *et al*., 2000; Faris *et al*., 2000; Salvo-Garrido *et al*., 2004). This preferred integration was found to be true in monocot species (Pedersen *et al*., 1997) and petunia (ten Hoopen *et al*., 1999).

During the integration of a transgene into the plant genome, a disruption may occur within the DNA and it is important to establish whether the disruption is contrary to an event that may occur during natural recombination mechanisms. Furthermore, the transgene site of integration must be clearly analysed to investigate whether this site is not an active gene-rich region, thus causing changes to biochemical pathways within the plant. Matzke and Matzke (1998) sequenced the regions flanking the transgene following the T-DNA insertion into the tobacco genome. The data revealed the frequent presence of motifs, and include microsatellite sequences, AT-rich sequences characteristic of matrix-attached regions (MARs), retroelements and tandem repeats. MARs have been said to be important for the expression of integrated reporter genes, the protection of transgenes from position effects, serve as the replication origin, as well as targeting transgene integration into the host genome (Morikawa *et al*., 2002). Several authors evaluated the junction regions in transgene loci and found genomic sequences that contained AT-rich MARs elements (Morikawa *et al*., 2002).

1.5.3. Gene expression profiling

Molecular marker technologies serve as more rapid and cost-effective methods for gene expression analysis. In potato, several molecular marker techniques have successfully been used for various research applications, such as cultivar identification, identification of genes for important agricultural traits, marker assisted selection and genetic linkage map construction (Gebhardt *et al*., 1991; Bryan *et al*., 2002; Ulrich and Dunemann, 2014).

Previously, gene expression analysis was performed using the Northern blot technique and this technique only allowed the analysis of a single gene per study (Eikhom *et al*., 1975). However, developments have facilitated analysis of differential gene expression, also known as transcript profiling, where the expression of a multitude of genes can be simultaneously analysed. Differential gene expression has been divided into two categories, namely closed and open architecture systems (Brent, 2000; Green *et al*., 2001). A closed system is one where the genes of interest are known and the genome from which the genes are derived has been well characterised (Green *et al*., 2001). On the other hand, open systems are those that do not require prior knowledge of the transcriptome, as well as the genome of origin.
Several methods were analysed prior to the execution of the research objectives to ensure that the appropriate method is used for optimal gene expression profiling. The methods that were studied include: Serial Analysis of Gene Expression (SAGE), Simple Sequence Repeats (SSRs), Inter-Simple Sequence Repeat (ISSR), Restriction Fragment Length Polymorphism (RFLP), microarray technology, Differential Display (DD), Arbitrarily Primed PCR (AP-PCR),

Representational Difference Analysis (RDA), Amplified Fragment Length Polymorphism (AFLP), quantitative reverse transcriptase real time PCR (qRT-PCR).

SAGE is a gene expression method which allows for quantification and analysis of genes with unknown sequences (Velculescu *et al*., 1995). This method employs two processes which entail, firstly, the production of short sequence tags (STTs) from cDNA followed by, secondly, linking and cloning of these tags for sequencing. The procedure begins with the synthesis of a double-stranded cDNA from mRNA using a biotinylated oligo (dT) primer. An anchoring four-base restriction enzyme is then used to cleave the cDNA. An example of the most commonly used anchoring enzyme is *Nla*III. The 3'-most region of the cleaved cDNA is then recovered with a cohesive 5'-terminus through binding to streptavidin-coated beads. The reaction mixture is then divided into two portions which are then mixed with two independent linkers for ligation to the cohesive 5'-terminus. The independent linkers are designed to contain type II enzyme, either *Fok*I or *BsmF*I, sites near the 3'-region of the anchoring enzyme sequence. The reaction mixtures are then restricted with the IIS enzymes, and the two portions are recovered and these products contain staggered ends. T4 DNA polymerase is then used to blunt the staggered ends and the two portions are mixed together and ligated. The disadvantage of this technique is that it requires high quality sequencing, since a small error may lead to sequence information loss. In addition, the technique does not cater for small variations in amplification efficiencies as this may also lead to loss of information. LongSAGE is a modified SAGE that enables transcriptome analysis of increased lengths which in turn improves the accuracy of annotating genes (Li *et al*., 2006; Zheng *et al*., 2011a).

SSRs, also known as microsatellites, are tandem short oligonucleotide repeat sequences flanked by conserved DNA sequences that can be used to obtain a DNA-based fingerprint of the study plant and has been said to be reliable and efficient (McGregor *et al*., 2000b; Coombs *et al*., 2004; Feingold *et al*., 2005; Sun *et al*., 2014; Zeinalabedini *et al*., 2014). Microsatellites have been seen as advantageous as they are simple to perform, low amounts of DNA are required, highly reproducible and the ability to detect high levels of polymorphism (Milbourne *et al*., 1997; McGregor *et al*., 2000b). During potato research studies, these markers were successfully used for the rapid identification of cultivars (McGregor *et al*., 2000b; Coombs *et al*., 2004). Another form of microsatellites, namely, ISSR, has been used on potato to determine the presence of somaclonal variation, as well as cultivar identification (Prevost and Wilkinson, 1999; Liu and Wu, 2013; Zhao *et al*., 2014).

RFLP was first used to construct the first molecular map of the human genome (Botstein *et al*., 1980). The developments led to the use of RFLPs to construct the first potato linkage

maps, as well cultivar analysis (Bonierbale *et al*., 1988; Gebhardt *et al*., 1989). Several authors have used Random Amplified Polymorphic DNA (RAPD) techniques for studies such as identification of disease resistant cultivars and the identification of clonal variants (Demeke *et al*., 1993; McGregor *et al*., 2000b; Pattanayak *et al*., 2002). This technique has also been used during cultivar characterisations of potato (Demeke *et al*., 1993).

A novel marker technique that has been an important application in potato research, including the identification of genotypes and the facilitation of potato breeding, is retrotranspon-based markers. The novelty of this technique stems from its ability to reveal extensive chromosomal distribution, as well as randomised genome distribution (Lightbourn *et al*., 2007; Spooner *et al.* 2007; Nováková *et al*., 2009).

Microarrays supply a global view of gene expression and are found in two forms; DNA-fragment-based and oligonucleotide-based microarrays (Lipshutz *et al*., 1995; Schena *et al*., 1995; Kehoe, 1999). These forms are a creation that is based on the robotic deposition of specific fragments of DNA on microscope slides at indexed locations. An estimated 10 000 spots/3.24 cm^2 can be arrayed, with the source of array fragments being either anonymous genomic clones, EST clones or ORF amplified DNA fragments. mRNA species detection is possible at a threshold of 1 part in 100,000 to 1 part in 500,000 (Schena *et al*., 1996; Ruan *et al*., 1998). The advantage of this technique is that a range of both weak and strong signals can be monitored on the same microarray, enabling the simultaneous analysis of a large number of genes. In addition, the technique allows for a pair-wise comparison of samples (Schena *et al*., 1995). Furthermore, since an oligonucleotide-based microarray is 20 to 25 nt, the hybridization reactions are more sensitive. However, a major disadvantage of this technique is that an accurate sequence database must be available to facilitate the construction of the microarrays, as well as a large amount of mRNA as starting material to perform the gene expression analysis (Kehoe, 1999).

However, all of the above techniques require substantial amount of sequence information of the study genome. However, at the beginning of this study, funding was limited and as a result, alternative gene expression techniques needed to be studied and investigated.

Gene expression analysis was first performed using DD and AP-PCR (Liang and Pardee, 1992; Welsh and McClelland, 1990). During DD, cDNA is synthesised from mRNA of each sample of interest, followed by amplification using a combination of anchored oligo-dT and random oligonucleotides. The obtained amplification fragments each represent a transcript or an expressed sequence tag (EST). The advantage of this technique is that it requires a small

amount of bioinformatics application during data analysis. However, its disadvantage is the high occurrence of false positives (Martin and Pardee, 2000). AP-PCR is a technique that entails two initial cycles at low stringency, followed by high stringency amplification, and the oligonucleotides used in the technique are designed to be arbitrary (Navarro and Jorcano, 1999). Different from DD, AP-PCR is normally used to analyse genomes rather than gene expression.

Subtractive hybridization of mRNA is one method that has been employed to identify mRNA that transcribe certain events within a cell, but the disadvantages of this method includes its technical difficulty, impracticality and time consumption (Wieland *et al*., 1990). A reduction in the number of genes analysed in a study is more favourable and simplify the technique employed for such (Hubank and Schatz, 1994). Differential display (DDRT-PCR) is a powerful tool that is used to identify and isolate expressed transcripts during a set time frame, as well as a comparative study between several mRNA populations (Liang and Pardee, 1992; Welsh *et al*., 1992). An advantage of the technique is its ability to reveal lower abundance transcripts (Guimaraes *et al*., 1995), but similar to substrative hybridization, the technique is time consuming and labour intensive (Ali *et al*., 2001).

Representational Difference Analysis (RDA) is a subtractive DNA enrichment technique that was designed to identify differences between two genomes without quantifying expression levels (Lisitsyn and Wigler, 1993). The technique was later modified by using cDNA as template to facilitate the the detection of rare transcripts (Gurskaya *et al*., 1996). cDNA-RDA utilises subtractive DNA enrichment in complementation with PCR amplification, where two cDNA populations are hybridized to analyse genes that are differentially expressed under set and differing conditions (Hubank and Schatz, 1994; Wallrapp and Gress, 2001; Konstantinov *et al*., 2005; Zhang *et al*., 2010). One population serves as the driver and the other is the tester. Two to three rounds of subtractive/ kinetic enrichment and selective amplifications are performed to determine the enrichment of differences between the two cDNA populations. The obtained difference products are sequenced and analysed to determine the difference in gene expression levels between the two genomes. A noted disadvantage of this technique is the high levels of labour it requires.

Amplified Fragment Length Polymorphism (AFLP) is a PCR-based technique that has been widely used for its advantages since it utilises PCR analysis on a small amount of DNA for the identification of various polymorphisms, (Zhang *et al*., 2012; Li *et al*., 2013; Lai *et al*., 2014; Thakur *et al*., 2014; Zeinalabedini *et al*., 2014). In potato, several applications have been found for AFLP and these include identification of potato cultivars, identification of the relatedness

of cultivars, analysis of the life cycle of potato, effect of gibberellic acid in plant development and evaluation of tuber organogenesis (Milbourne *et al*., 1997; Bachem *et al*., 2000a; McGregor *et al*., 2000b; Bachem *et al*., 2001; Trindade *et al*., 2003). However, the use of mRNA expression analysis through cDNA-AFLP has been greatly utilised in potatoes, as they afford the researcher to target coding regions, as well as to facilitate gene expression analysis that lead to the identification of genes involved in different biological processes (Reijans *et al*., 2003; Ritter *et al*., 2008).

QRT-PCR has been described as a sensitive, highly specific and broad range technique that can be used for gene expression analysis (Bustin *et al*., 2005). This technique offers researchers the ability to investigate rare transcripts, as well as analyze multigene families. However, researchers can only benefit from the effectiveness of this technique if proper internal controls are included. These controls, also known as reference genes, normalize the expression analysis, since they are consistently expressed in tissues of interest under varying experimental treatments (Dombrowski and Martin, 2009).

Gene expression forms a crucial part of the formulated research study, thus understanding the dynamics of the various techniques was important in selecting the appropriate techniques for the realisation of the set objectives. In this study, the test crop for the genomic evaluation of the possible unintended and unexpected effects of transformation was the *Mdpgip1* transgenic potato (Maritz, 2002; Gazendam *et al*., 2004). At the inception of the research, the comprehensive potato sequence was not available as the genome was still being sequenced at that time. In addition, a microarray chip for potato was also not available to facilitate gene expression analysis. Furthermore, the project budget was not sufficient to conduct research using the newer technologies that were available at inception such as next generation sequencing (NGS). The limited budget, the absence of a suitable microarray chip as well as limited potato genome sequence information led as well as to the selection of alternative transcriptome profiling methodologies that were able to overcome these initial short comings. The techniques that were selected for gene expression profiling in this project were cDNA-RDA and cDNA-AFLP. Both techniques can be used with ease as prior knowledge of the potato genome is not needed for execution. Each method has its advantages and limitations. Thus, using them in complement would ensure optimum results for investigating differential gene expression analysis. cDNA-AFLP would allow for the evaluation of a large pool of genes differentially expressed between the transgenic and the traditional counterpart. The large pool of differentially expressed genes obtained from cDNA-AFLPs would then be filtered using the enriching subtractive cDNA-RDA which would also allow for the detection of rare transcripts (Kok *et al*., 2007; Wang *et al*., 2007). To validate the results obtained from these techniques,

some differentially expressed genes would be selected and further scrutinised using qRT-PCR (Costenaro-da-Silva *et al*., 2010).

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