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Detection of Genetically Modified Foods

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Detection of Genetically Modified Foods

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Declaration

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Acknowledgments

This thesis is dedicated to Noel O'Reilly – none of this would have been possible without his help and good humour when things broke down/blew up. Tea-time will never be the same again.

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Finally, to my parents and family. Twenty years later and this is almost it!! Thank you for everything - I couldn't have done it alone.

Abbreviations

A(a)	adenine
AP	Alkaline Peroxidase
APH(3')II	Aminoglycoside 3' Phosphotransferase II
ATP	Adenosine Triphosphate
bp	Base Pairs
BSA	Bovine Serum Albumin
BT toxin	*Bacillus thurlingensis* toxin
C(c)	Cytosine
CaMV	Cauliflower Mosaic Virus
CAT	Chloroamphenicol Acetyltransferase
CTAB	Cetyl Trimethyl Ammonium Bromide
dATP	deoxyadenosine Triphosphate
dCTP	deoxycytosine Triphosphate
DEAE	Diethylaminoethyl
dGTP	deoxyguanisone Triphosphate
DIG	Digoxygenin
DIG-11-dUTP	Digoxygenin-11-deoxyuridine 5' triphosphate
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside Triphosphate
dTTP	deoxythymidine Triphosphate
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EPSPS	5-enol-3-pyruvylshikimic Acid Phosphate Synthase
EU	European Union
G(g)	Guanine
GM	Genetically Modified
GUS	β-glucuronidase
HRP	Horseradish Peroxidase

HTT/PMS	Thiazol blue/Phenazine methosulphate
K bp	Kilo base pairs
ME	Mercaptoethanol
mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
nm	Nanometres
NPT II	Neomycin Phosphotransferase II
ORI	Origin of Replication
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PET	Polyethyleneimine
PG	Polygalacturonase
P-nos	Nopaline Synthase Promoter Gene
PVP	Polyvinyl Pyroline
PVX	Potato Virus X
PVY	Potato Virus Y
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
T(t)	Thymine
T-DNA	Transfer DNA
TE	Tris/EDTA buffer
TMB	3,3',5,5'-tetramethylbenzidene
UV	Ultraviolet

Table of Contents

Abstract

In recent years, Genetically Modified (GM) foods have become increasingly common on our supermarket shelves. Consumer concerns regarding their safety have prompted codes of practice and legislation requiring labelling of all GM-food-containing products. Labelling requires some means of verification. There is no simple means of detecting GM food and until recently, there were no tests available. The object of this study was to develop a simple, rapid and user-friendly method of detecting genetically modified foods.

This study concentrated on the detection of GM tomatoes, using a commercially available tomato paste. The method of choice was the Polymerase Chain Reaction (PCR) targeting the selectable marker gene Neomycin Phosphotransferase II (NPT II). The selectable marker gene confers resistance to the antibiotic kanamycin, and is used to detect newly transformed plants in the laboratory. NPT II has commonly been used as a selectable marker and therefore can be used to test for many species of transgenic plants.

A PCR method to detect NPT II was developed and applied to genetically modified tomato paste. Commonly used plant DNA extraction methods proved unsuitable and an extraction method based on microwave treatment of the paste was developed. This increased both the sensitivity and reproducibility of the PCR method. It was also attempted to develop a method to detect genetically modified soy using PCR, by targeting the cauliflower mosaic virus promoter gene.

Chapter 1: Introduction

1.1 Introduction

Man has selectively bred crops for tens of thousands of years. Using classical breeding methods he has increased yields, disease resistance and improved the taste of his food. However, this was a slow, inaccurate and laborious process involving repeated hybrid crosses and selection. Hybrid formation was limited to crossing the entire complement of genes of two individuals of opposite sex of the same, or closely related species only.

Genetic engineering, which allows the isolation of a gene from any organism, and the subsequent transfer of that gene to another organism, has been possible since the 1970's. This technology allows the sexual incompatibility barrier between species to be crossed and forms the backbone of the burgeoning biotechnology industry. Recombinant proteins such as human insulin, interferons and vegetarian rennet have been available for many years. Recently, the food industry has been revolutionised by the development and commercialisation of Genetically Modified (GM) foods.

GM plants have a gene for a beneficial trait inserted into their genome by genetic engineering. Genetically modified food looks and tastes like non-modified food. This leaves the food producer and the consumer without a way of distinguishing one from the other. It will therefore be necessary to develop a reliable, technically simple and cost-effective method of detecting modified food (Boyce *et al.*, 1998)

Genetic engineering is not a new technology, having been established now for over twenty-five years. However, the idea of genetic engineering has only recently entered the public domain, where it is poorly understood. Genetically modified food plants have particularly focused the public's attention on this revolutionary technology.

Some of the concerns relating to the growth and consumption of genetically modified foods include (Snow and Palma, 1997):

- The safety and allergenicity of the foreign proteins produced by GM plants. There are concerns that the insertion of antibiotic resistance genes into plants could compromise the antibiotic as a therapeutic agent in humans.

- Bacterial exchange of genetic material by conjugation. There is the possibility that bacteria in the human gut, or soil biota, could acquire the gene for kanamycin resistance from ingested food and render the antibiotic ineffective due to widespread resistance.

- There is concern that genes from genetically engineered plants could spread into closely related wild species in the field by cross-pollination and produce 'Superweeds'. This is especially relevant in plants engineered to be herbicide and insect resistant.

- Engineering for herbicide resistance may also result in the increased use of a particular herbicide - glyphosate. There are valid economic concerns about the monopoly held by large biotechnology companies, who produce both the seed and the herbicide.

- Seeds of GM crops are only available from the seed companies that produce them and many GM plants are now being engineered to be sterile. The situation could arise where farmers would lose the right, or the ability to save seed from their crop for replanting.

- A predicted result of the acceptance of GM food would be a reduction in the planet's biodiversity and an increase in monoculture. Millions of hectares of the planet are currently planted with the same variety of crops, and local varieties are being lost.

- There are religious and ethical concerns also. Biotechnology is speeding up evolution immeasurably as the molecular clock is ticking faster. There is the view that man should not 'be playing God' with the planet's flora and fauna, and should allow the planet to evolve at its own pace.

Consumer concerns have culminated in the introduction of European Union (EU) directives to control the release and labelling of GM foods and food products. Council Directive 90/220/EEC (Deliberate Release of Genetically Modified Organisms into the Environment), EU Regulation 258/97 (Concerning Novel Foods and Novel Food Ingredients) and EU Regulation 1139/98 (Concerning the Compulsory Labelling of Certain Foodstuffs Produced from Genetically Modified Organisms) govern the use of genetically modified organisms in foods (Pierce, 1997; Food Safety Authority of Ireland, 1999). In the future, as these directives become legislation in the member countries, it will be necessary to have a means of detecting GM plants and food ingredients entering the food chain.

The objective of this research was to develop a reliable, user-friendly and rapid method to detect genetically modified foods using tomato paste and soy as suitable models. EU legislation demands that all foods or food products containing genetically modified foods should carry the appropriate labels. This not only applies to foods approved and produced within the EU, but also to foods imported from outside the EU – especially imports from the US, where genetically modified foods are not identified.

The following sections will outline the modern technology used in the genetic modification of plant foods.

1.2 Plant Transformation

Transgenic plants can have a gene from any source inserted into their genome. These genes are fully functional (Uchimiya *et al* 1989). Advances, both in plant tissue culture and recombinant DNA techniques, have allowed plant transformation to become common and more successful than before. Many plant species can now be transformed and some are available commercially – the first, and most famous example being the FLAVR SAVR™ tomato. There are several methods available for genetic modification of cells and common methods transforming plant cells are reviewed briefly below.

1.2.1 *Agrobacterium tumefaciens*-mediated transformation

Agrobacterium tumefaciens mediated transformation is the most commonly used method of DNA transfer into plants. *A. tumefaciens* is a soil bacterium that infects many dicotyledenous plant species in the wild, causing a condition known as crown gall disease. This disease is characterised by tumours that appear on the plant at the site of a wound. The tumour is caused by the bacterium transferring a piece of DNA (T-DNA) into the cells at the site of the wound. This T-DNA is contained on a large plasmid called the Ti-plasmid. T-DNA is stably integrated into the genome of the cell and expression of T-DNA encoded genes results in tumour formation (Fisk *et al.*, 1993).

In plant transformation, the genes responsible for crown gall disease are removed and can be replaced by any other desirable gene (see *Figure 1*). The 'new' gene may then become stably integrated into the host plant genome after transfection. For this DNA transfer, only the T-DNA borders and T-DNA border flanking sequences are required. This strategy permits the gene of interest to be inserted into the plant (An *et al.*, 1985). However, in contrast to the above, little success with gene transfer in monocotyledonous plant species has been achieved, as the low infection rate and specificity makes transformation difficult (Christou, 1996).

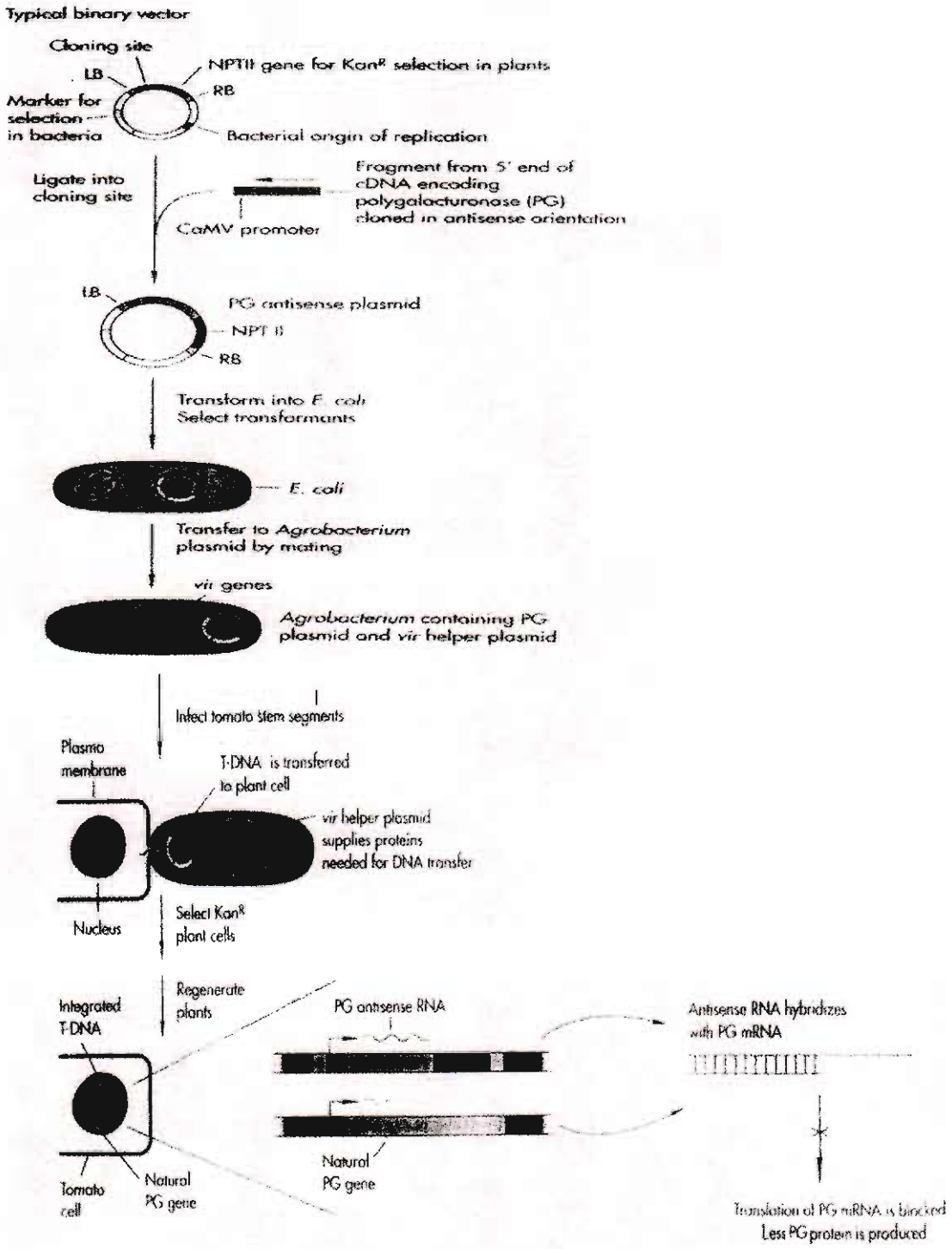


Figure 1: Schematic diagram of Agrobacterium tumefaciens-mediated transformation.

1.2.2 Direct Gene Transfer

Transformation by Direct Gene Transfer is usually less efficient when compared to infection with the Ti plasmid of *A. tumefaciens*. However, these methods are important when dealing with plants that are difficult to transform. A brief summary of the common strategies are outlined below.

1.2.2.1 Micro-Particle Bombardment

Small tungsten or gold particles (1-4 μM in diameter) carrying adsorbed DNA or RNA are accelerated at high velocity (1,000 to 2,000 ft/sec) by a specially designed particle accelerator (Klein *et al.,* 1987). These microparticles are sufficiently small to rapidly pass through cell walls and membranes without causing damage, and carry the nucleic acids into the nucleus or chloroplast of the plant (Uchimiya *et al.,* 1989). This method has been found to be successful for many species that are recalcitrant toward transformation using *A. tumefaciens* (Ritala *et al.,* 1994). This method is highly efficient, as large numbers of cells can be bombarded simultaneously with low mortality rates post-transformation (Sanford *et al.,* 1987).

1.2.2.2 Polyethylene Glycol (PEG) Mediated Gene Transfer

Polyethylene Glycol has been used to permeablise plant cells, improving their susceptibility to transformation. This method has been found to be especially effective for protoplast transformation - where the cell wall has been enzymatically digested - allowing foreign plasmid DNA to be inserted (Uchimiya *et al.,* 1987).

1.2.2.3 Electroporation

High voltage electrical pulses are used to reversibly permeablise the lipid bilayers of cell membranes (Fromm *et al.,* 1987). Since the plant cell wall is an effective barrier to the entry of DNA, protoplasts are used (Fisk *et al.,* 1993). An electrical pulse is applied to a solution of protoplasts and foreign DNA resulting in the transformation of the desired cells. This method is useful for transforming monocotyledonous plant cells such as maize (Fromm *et al.,* 1986; Rhodes *et al.,* 1988) and rice (Toriyama *et al.,* 1988).

1.2.2.4 Microinjection

Both tissue culture cells and intact plant organs, such as leaves, can be transformed using microinjection. DNA is directly injected into the cell nucleus or organelle using a specially designed microcapillary tube (Uchimiya *et al.,* 1987). The method is not commonly used for plant genome modification, as it is both slow and labour-intensive.

1.3 Gene Constructs

To ensure successful DNA transfer into the plant genome it is necessary to assemble a gene cassette with the essential features permitting both integration and expression of the foreign DNA (*Figure 2*). Furthermore it is also necessary to include a selectable marker gene allowing the identification of any newly transformed cells in the laboratory. Typical selectable markers include genes that confer antibiotic resistance. Among those commonly used is the Neomycin Phosphotransferase II (NPT II) gene that confers resistance to aminoglycoside antibiotics, such as kanamycin.

Consumers have expressed concerns about such selectable marker genes, and biotechnology companies have attempted to address these concerns. Some newly-available GM plants are self-selective without the incorporation of a selectable marker gene e.g. Glyphosate resistance is self-selecting as all non-transformed cells will die in the presence of the herbicide. A new approach involves using genes from honeybees which target both bacteria and fungi, and are also self-selecting (Coghlan, 1999). A terminating sequence is also included to 'switch off' transcription of the gene.

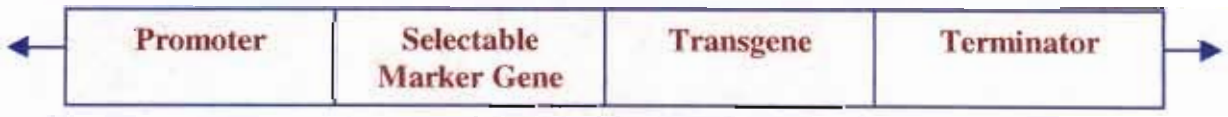
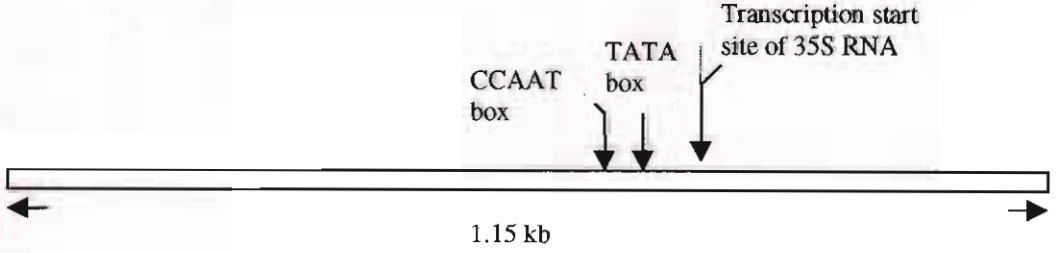


Figure 2: A representative diagram of a typical gene construct. The 'Transgene' is the gene inserted into the plant that confers the introduced trait on the plant.

1.3.1 Promoter Sequences

The most frequently used promoter sequence is the Cauliflower Mosaic Virus 35S promoter (Hemmer, 1997). The cauliflower mosaic virus is a double stranded DNA virus containing two separate promoters – producing 19S and 35S transcripts respectively (Kay *et al.,* 1987). The 35S promoter is active in many species and can be used to express foreign genes in transformed plants. Initiation of transcription is dependent on proximal sequences that contain a TATA element (see *Figure 3*), while the rate of transcription is controlled by sequences contained in 300 bp of upstream DNA (Odell *et al.,* 1985). Promoters basically 'switch on' expression of the inserted trait gene. Another common promoter used in transgenic plants is the Nopaline Synthase (P-nos) promoter from *Agrobacterium tumefaciens* (Ulian *et al.,* 1996; Umbeck *et al.,* 1989). Transcription initiation is one of the key steps involved in gene regulation (An *et al.,* 1986).

Figure 3: 1.15 kb fragment of the CaMV gene showing the location of the CCAAT box and the TATA box.



1.3.2 Terminator Sequences

Terminator sequences halt transcription at the 3' non-coding end(s) of genes. The most frequently used terminator for genetically engineered plants is the nos 3' terminator sequence, which is isolated from the *A. tumefaciens* nopaline synthase gene (Parkes, 1999; Hemmer, 1997).

1.3.3 Selectable Marker Genes

A selectable marker gene must be expressed in a wide variety of host backgrounds and should also confer a selectable change on transformants. Plant cells are susceptible to aminoglycoside antibiotics (non-resistant green cells are bleached) therefore, incorporation of aminoglycoside resistance into the genome provides an easy and effective method of transformant identification (Herrera-Estrella *et al.,* 1983). Selectable marker genes found in genetically modified plants include the Neomycin Phosphotransferase II (NPT II) gene, also known as aminoglycoside 3'-phosphotransferase II (*aph*(3')II). This gene confers resistance to the antibiotics; kanamycin, neomycin, paromycin, ribostamycin, butirosin and gentamycin B (Matsuhashi *et al.,* 1975). The 1.25 kb ORI of this gene encodes an enzyme of 280 amino acids. The latter gene was originally cloned from transposon Tn5 of *Escherichia coli.* (Beck *et al.,* 1982; Redenbaugh *et al.,* 1994). Tn5 is a mobile DNA element of 5.4

kb (Jorgensen *et al.,* 1979). The complete nucleotide sequence was elucidated by Beck *et al.,* 1982.

Aminoglycoside antibiotics work by binding to the ribosomes of susceptible bacteria and interfering with protein translation. Mutations resulting in aminoglycoside resistance either prevent the drug binding to the bacterial ribosome, or prevent cross-membrane transport of the drug into the cell. In the case of NPT II, the modified antibiotic cannot bind to the ribosome and therefore it is unable to inhibit protein synthesis within the cell. The antibiotic is modified (*Figure 7)* by phosphorylation – kanamycin A is inactivated by phosphorylation of the 3' hydroxyl group (OH) of its 6-deoxy-6-aminoglucose-1-alpha sugar residue (Foster *et al.,* 1983; Norelli *et al.,* 1993).

Because of the similarity between plant chloroplast and bacterial ribosomal DNA, antimicrobial agents are fully effective in plants. This is based on the Endosymbiotic Theory of Eukaryote Evolution, which suggests that the ancestors of eukaryotic cells formed mutually beneficial 'symbiotic consortiums' with one or more species of prokaryotic cells. Typically, susceptibility is evident due to the bleaching of plant cells, as the antibiotic targets chloroplasts.

Several other selectable markers that can be used include

- Chloramphenicol acetyltransferase (CAT) – (Pietrzak *et al.,* 1986)
- *E. coli* β-glucuronidase (GUS) has a broad host range, positive genetic selection and is detected by a simple assay with low background interference (Datla *et al.,* 1991)

- Hygromycin B resistance is commonly used in mammalian cells (Sorenson *et al.*, 1992).
- Due to consumer resistance to the use of antibiotic resistance non-antibiotic selectable markers are being developed – such as the green fluorescent protein and *msrA1* (Davis and Vierstra, 1998; Coghlan, 1999).

In the case of genetically modified soy beans that carry the gene for resistance to the herbicide glyphosate, herbicide resistance itself can be used as a selectable marker gene. The gene for herbicide tolerance has been inserted into several plant species making it one of the main modifications of GM plants.

1.3.4 Negative Selectable Markers

Antisense RNA technology also has certain attractive features as a modifying principle. Antisense RNA technology is designed to prevent the production of a protein from a targeted gene (see *Figure 4*). The positive selectable marker gene is integrated within the genomic flanking sequences of the plant while the antisense gene is integrated outside the homologous region. Random integration or non-homologous recombination occurring after transformation causes integration of the antisense gene into the plant genome and non-expression, or decreased expression of the selectable marker gene. Therefore, only homologous recombinants will survive screening (Xiang and Guerra, 1993).

Some modifications are self-selecting, for example, soy modified to be herbicide resistant (Round-Up) contains the glyphosate-resistance gene 5-enol-3-pyruvylshikimic Acid Phosphate Synthase (EPSPS). A selectable marker gene is unnecessary in this case, as non-transformants (which do not contain the resistance gene) will die in the presence of the herbicide. This is used to select for newly transformed plants in the laboratory.

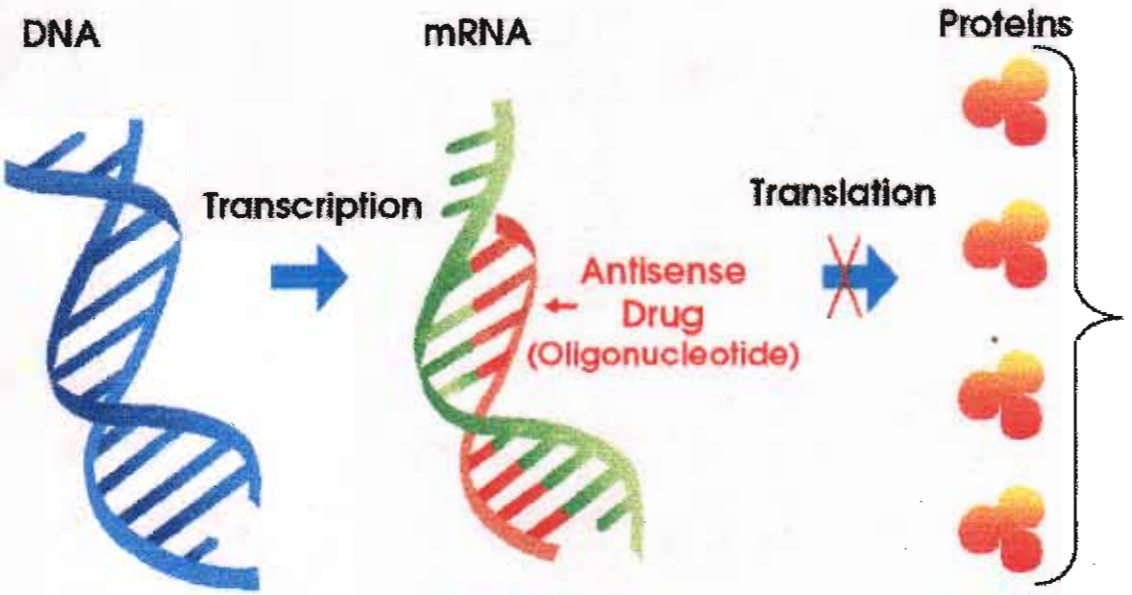


Figure 4: Antisense Technology. Gene expression is altered by the binding of an antisense oligonucleotide to a target gene, preventing protein translation from that gene.

1.4 Examples of Engineered Plants

Advances in transformation technology allow more plant species to be transformed. Consequently, GM plants are now commercially available as food products and ingredients in the EU (*Table 1*). The major crop plants that have been engineered to date, include banana, barley, bean, canola, cassava, maize, cotton, papaya, peanut, poplar, potato, rice, soybean, squash, sugarbeet, sugarcane, sunflower, tomato and wheat. (Christou, 1996).

Plant	Engineered Traits	Gene
Oilseed Rape	Male Sterility	Glufosinate-bar gene
	Herbicide Tolerance	Glufosinate-pat gene
Chicory	Male Sterility	Glufosinate-bar gene
	Herbicide Tolerance	Glufosinate-pat gene
Maize	Insect resistance	BT toxin
	Herbicide Tolerance	Glufosinate-bar gene
		Glufosinate-pat gene
		Glyphosate-CP4-EPSPS
Soybean	Herbicide Tolerance	Glyphosate-CP4-EPSPS

Table 1: GM plants approved by the EU, with a list of added traits and the genes that confer them (Biotechnology Club Bulletin, 1998)

In addition to commercially available plants, many transformed plants are used purely for research, or have not yet been approved for consumption by the public. Some examples include: tobacco (*Nicotiana tabacum*) used mainly as a research tool (Ye *et al.,* 1996; Shao *et al.,* 1995, Matzke *et al.,* 1989), potatoes (*Solanum spp*) transformed to confer resistance to the common potato viruses; Leuteovirus, Potato leaf roll virus (PLRV), potato virus X (PVX) and potato virus Y (PVY) (Tacke *et al.,* 1996; Smith *et al.,* 1995) and rice which was transformed to confer insect resistance (BT toxin), increased nutritional value and increased iron content or virus resistance. Other selected features include genes encoding proteinase inhibitors which prevent insects digesting plant material (Duan *et al.,* 1996). Proteinase inhibitors are destroyed by cooking, and therefore the plants are still edible by humans.

1.5 Genetically Modified Tomatoes

A genetically modified tomato was the first GM plant food to become commercially available. Since the present study has focused on GM tomato products they will be considered in detail here. Several genetically modified tomatoes have been developed. *Table 2* shows the two major GM tomatoes together with their modified characteristics.

Plant	Company	Modification	Inserted Gene
Tomato (FLAVR SAVR™)	Calgene	Delayed fruit ripening	Antisense polygalacturonase NPT II
Tomato (Processed)	Zeneca	Thicker Skin Delayed fruit ripening	Truncated-polygalacturonase NPT II

Table 2: An overview of GM tomatoes available in the UK. (Hemmer, 1997)

The FLAVR SAVR™ tomato – developed by Calgene – was the first transgenic plant to become available to the public for consumption. The US Food and Drug Administration approved it in 1994. Assessments proved that the constructed tomato was safe and just as nutritious as non-GM tomatoes, being unchanged apart from the intended effects of the inserted gene cassette (Redenbaugh *et al.,* 1994). The FLAVR SAVR™ tomato was engineered using antisense RNA technology. The enzyme polygalacturonase (PG) breaks down pectin in the cell walls of tomatoes causing the fruit to soften over time (Bird *et al.,* 1988). The antisense mRNA transcribed from the antisense PG gene incorporated into the gene cassette binds to the host-expressed mRNA and thereby inhibits the production of the polygalacturonase enzyme, slowing down the ripening of

the tomato fruit (Redenbaugh *et al.,* 1994). Transformation was achieved using the *A. tumefaciens* model and the selectable marker NPT II.

Schuch *et al.,* 1991, reported that tomatoes transformed and expressing anti-PG RNA gene segment benefit from a longer shelf life and better tolerance for transportation compared than non-transformed control fruit. It was also observed that the transformed fruit had lower levels of PG activity throughout the ripening process and firmness was comparable to control fruit.

A second example of an engineered tomato consisted of a tomato paste developed by Zeneca Plant Sciences processed and sold by J Sainsbury Ltd. (UK). In this case the tomato plant was engineered by *A. tumefaciens* to contain a truncated form of the polygalacturonase enzyme as outlined in *Figure 5*. The promoter used in the construction was the CaMV 35S promoter with NPT II as the selectable marker. This gene cassette produced a tomato with a thicker than normal cell wall – which is considered ideal for processing purposes as this produces a higher solids content yield. A schematic diagram explaining the processing steps involved is shown in *Figure 6*.

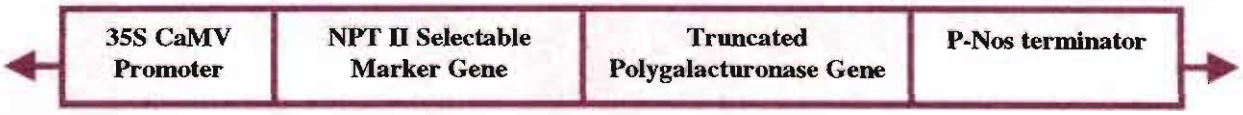


Figure 5: A schematic diagram of the gene cassette contained in the genetically modified tomato paste sold by J Sainsbury Ltd (UK)

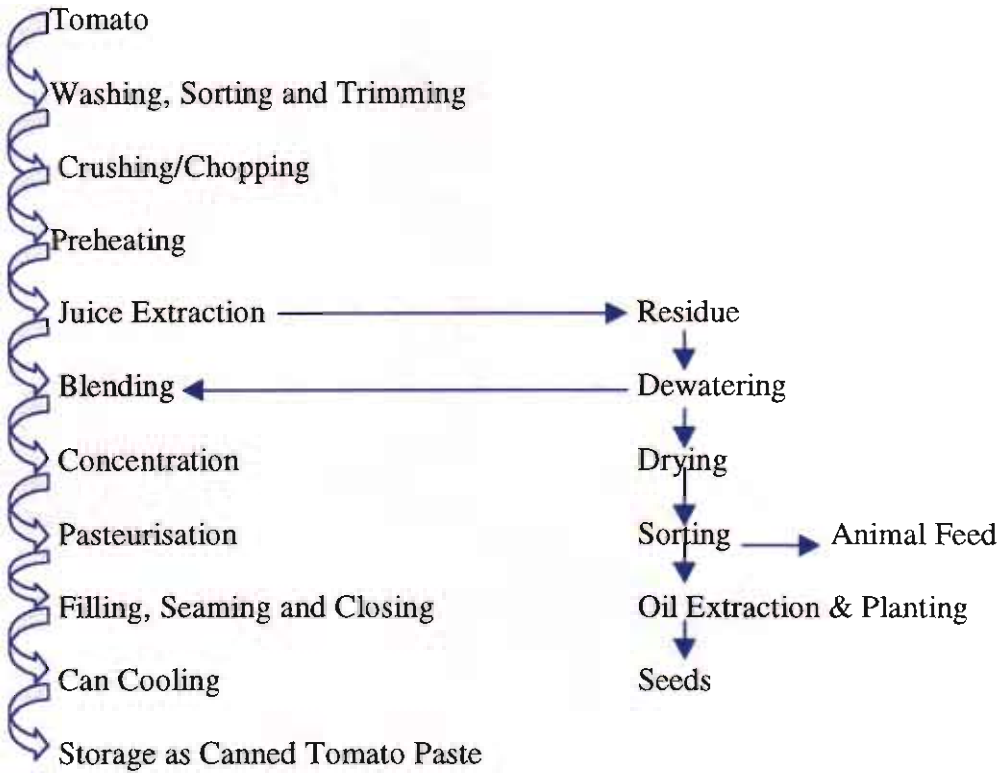


Figure 6: *Diagram from Hayes et al., 1998 describing the production of tomato paste. Chopped tomatoes are preheated to 60°C to produce products with low viscosity e.g. tomato juice. Chopped tomatoes are preheated to 90°C for products requiring high viscosity.*

1.5.1 Nucleotide Sequence of the NPT II gene

The NPT II gene sequence used in the GM tomato paste sold by J Sainsbury is that initially published by Beck *et al.*, (1982), and is available in GenBank (Accession number V00618).

1.6 Detection of Transgenic Plants

The transgene cassette inserted into GM plants destined for human consumption has been described (*Section 1.5).* The key to distinguishing a GM food from its unmodified counterpart depends on the ability to detect the presence of these genes or their protein products in the foodstuff. Of course, during the process of developing transgenic plants these genes had to be monitored. However, such methods could be based on portions of the plant not available to the consumer. Thus, one could check for a transgenic tomato plant by assaying the levels of transgenes or transgene enzyme products in the leaf and stem of the tomato plant. However, a consumer or food factory usually buys tomatoes without any leaf or stem attached and can only carry out analysis on the fruit.

The analysis becomes more difficult when the food has been processed in some manner. This is the case with the tomato paste, which is subjected to extensive heat treatment during processing resulting in the irreversible denaturation of proteins. This feature demands that detection of the transgenic plants be at the DNA level.

The situation becomes more complicated still when it is considered that tomato paste might be a component of a more complex food such as a pizza or pasta sauce. Complex foods such as these are increasingly being imported into Ireland. The complex food might also contain other GM material such as GM soy or GM maize. It is instructive to consider the case of the NPT II selectable marker gene, which confers antibiotic resistance on transgenic plants. This is a widely used marker gene in transgenic plants and it expresses the enzyme neomycin phosphotransferase. It has frequently been the basis for the development of methods to detect GM plants. A brief review of NPT II detection strategies is presented below.

1.6.1 Neomycin Phosphotransferase II (NPT II) Detection Methods

Neomycin Phosphotransferase II (NPT II) catalyses an ATP-dependent phosphorylation of the 3' hydroxyl group of the aminohexane component of the aminoglycoside structure (Goldman and Northrup 1976; *Figure 7)*, thus conferring resistance to these antibiotics. The NPT II gene has been commonly used as a selectable marker gene in genetically modified plants and therefore, methods for detecting NPT II can be applied to the detection of genetically modified plants containing the gene.

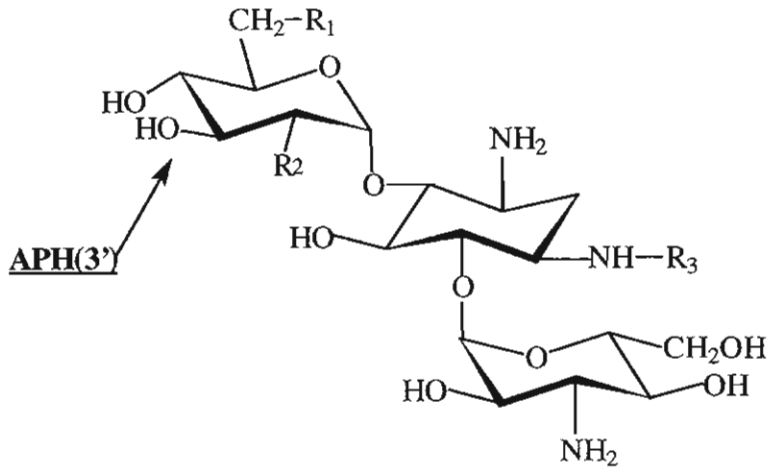


Figure 7: The Structure of Kanamycin. The site of Phosphorylation of Kanamycin by Neomycin Phosphotransferase II is indicated by the arrow.

1.6.1.1 Spectrophotometric Detection Methods

In transgenic food plants that have not undergone substantial processing the activity of neomycin phosphotransferase may be detected by enzyme assay as described by Goldman and Northrup (1976). The method links ADP production to pyruvate kinase and lactate dehydrogenase, and measures aminoglycoside phosphorylation as a function of NADH oxidation at 340 nm. Perlin *et al.*, 1988, modified the latter method for use in a spectrofluorometer by the inclusion of a chromogenic substrate.

1.6.1.2 Protein Determination Methods

Herrera-Estrella *et al.,* (1983) and Reiss *et al.,* (1984) developed a method to detect the activity of NPT II in plant samples by subjecting the protein to denaturing polyacrylamide gel electrophoresis (PAGE) separating it from other background proteins. The polyacrylamide gel was then covered by a 1% agarose gel containing kanamycin and [γ-^{32}P]ATP. The enzyme converted kanamycin into its [γ-^{32}P] labelled derivative. This was then immobilised on blotting paper and visualized by autoradiography. McDonnell *et al.,* (1987) further modified this method by eliminating the gel electrophoresis step and incubating the radiolabelled ATP with unlabelled ATP to decrease background signals.

Cabanes-Bastos *et al.,* (1989) detected NPT II-linked activity in tobacco leaves and calli. Polyethyleneimine (PET) ion-exchange plates were used to separate radiolabelled kanamycin phosphate from the other components of the reaction mixture. The radiolabelled kanamycin phosphate was then detected directly by autoradiography. Later Staebell *et al.,* (1990) further modified the method by adding fatty-acid free BSA protein to the reaction mixture to stabilise enzyme activity.

Peng *et al.,* (1993) published a method for assaying the NPT II protein without an initial protein extraction step. Cut samples – leaves, calli or even cells – were incubated directly in a microtitre plate with the reaction components. This reaction mixture was then assayed directly for NPT II by using the "dot-blot" method of McDonnell *et al.,*,

1987. All of these methods are only applicable to foodstuffs that have not been subjected to processing or cooking, which would normally inactivate the activity of NPT II.

1.6.1.3 Immunology-Based Methods

Rogan *et al.,* (1992) reported an Enzyme Linked Immunosorbent Assay (ELISA) to detect NPT II in genetically modified cotton. Anti-NPT II antibodies were immobilised onto microtitre plates and allowed to bind NPT II. A horseradish peroxidase (HRP)-labelled anti-NPT II antibody was then added which bound to any NPT II present. The substrate for HRP (3,3',5,5'-tetramethylbenzidene – TMB) was later added and the plate allowed to incubate in the absence of light. Absorbance at 450 nm was then measured using an automatic plate reader.

Platt *et al.,* (1987) developed a "dot-blot" method to detect NPT II activity in crude cell extracts of tobacco and cotton calli. Crude cell extract was applied to a non-denaturing polyacrylamide gel. After washing, the gel was overlaid with a 1% agarose gel containing radiolabelled ATP and the kanamycin sulphate substrate. The agarose gel was blotted onto Whatman P81 filter paper and the radiolabelled ATP present was quantified using scintillation counting. A dot assay of NPT II activity was also performed. Radiolabelled ATP, crude cell extracts and kanamycin/neomycin sulphate were reacted together in a microtitre plate and the mixtures then blotted onto P81 filter paper. Autoradiography was then performed on the filter to detect ^{32}P labelled kanamycin. This method was capable of detecting 0.01ng NPT II protein in solution.

More recently, Amorosa and Gutkind (1998) developed a test to distinguish two different aminoglycoside phosphotransferase genes. Bacterial strains carrying aminoglycoside phosphotransferase isozymes were biochemically screened based on the scheme outlined in *Figure 8*.

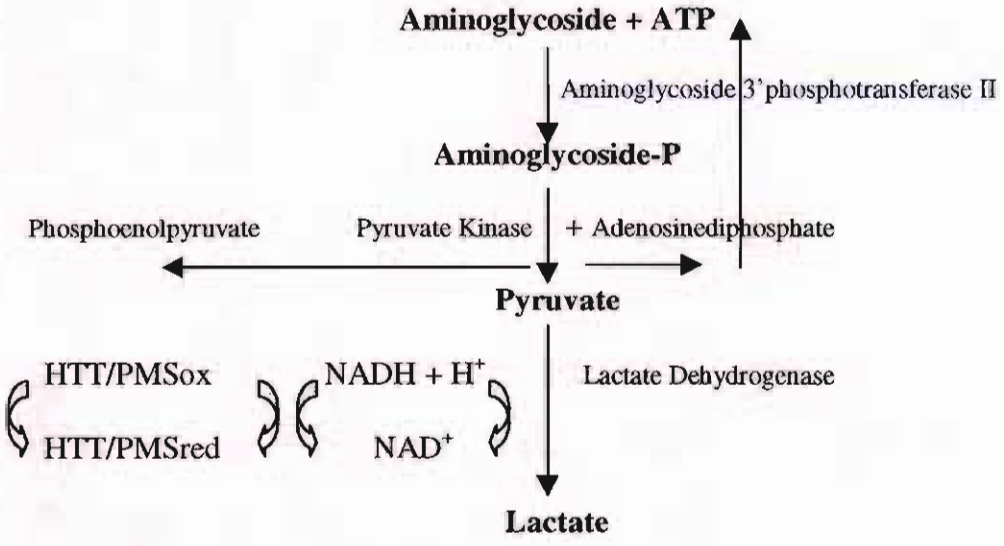


Figure8: *A representation of the reaction mechanism used as a basis for the detection of the NPT II isoenzyme in bacteria.*

Isoelectric focusing was used to separate the enzymes initially. An agar overlay was prepared containing NADH, pyruvate kinase, lactate dehydrogenase, phosphoenol pyruvate, ATP and kanamycin sulphate. The chromogenic indicator, Thiazoyl blue™ – phenazine methosulphate (HTT/PMS) was used in a colorimetric method to detect NADH uptake. Samples were placed on the agar plate and incubated at 37°C. TMM/PMS was then added and incubated at room temperature until a colour-change was observed. A blue colour indicated a positive result for APH.

As with the direct spectrophotometric methods, the use of immunological techniques for NPT II detection depended on acquiring an intact protein that was not so denatured as to be unrecognisable by the antibodies used in the assay. This limits the usefulness of such methods to foods that have not been processed.

1.6.1.4 Spraying

Weide *et al.*, (1989) published a method of screening tomato transformants post-germination. The plants, both transformed and non-transformed, were germinated for three weeks in a greenhouse. They were then sprayed with a solution of kanamycin sulphate. It was observed that non-transformed plants developed chloritic spots due to direct contact with the antibiotic solution and were easily identifiable. The assay was non-destructive as only leaves, which were actually in contact with the antibiotic, were bleached, and any further growth was unaffected. It is a suitable method for screening large numbers of germinants, but requires whole plants and takes several weeks.

As with the previous methods, this methodology does not lend itself for use with processed or cooked foods. It is clear that, when processed foods are considered, methods based on detection of transgene DNA represent attractive targets. Many such methods are available, however the most common and convenient ones are based on the polymerase chain reaction (PCR).

1.7 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) was developed by Kary Mullis of the Cetus Corporation in the 1980's (Mullis and Faloona, 1987). PCR utilises enzyme-mediated *in-vitro* methods of DNA polymerisation to amplify nucleic acids.

PCR consists of repeated cycles of Denaturation, Primer Annealing and Primer Extension (*Figure 9*). The template DNA used is a segment of double stranded DNA that has been separated into single strands by heating. Pairs of DNA primers are used to flank the region of DNA to be amplified. The primers are each highly specific and complementary to one of the DNA strands.

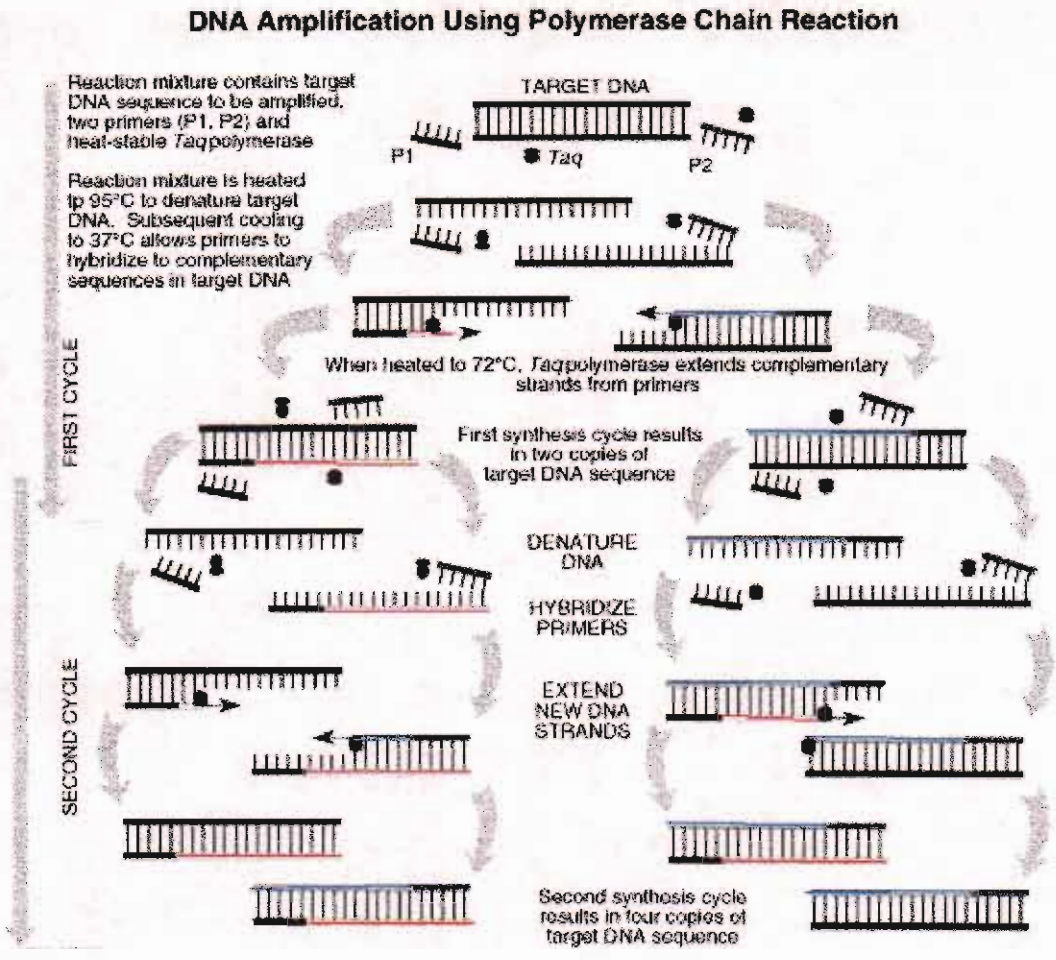


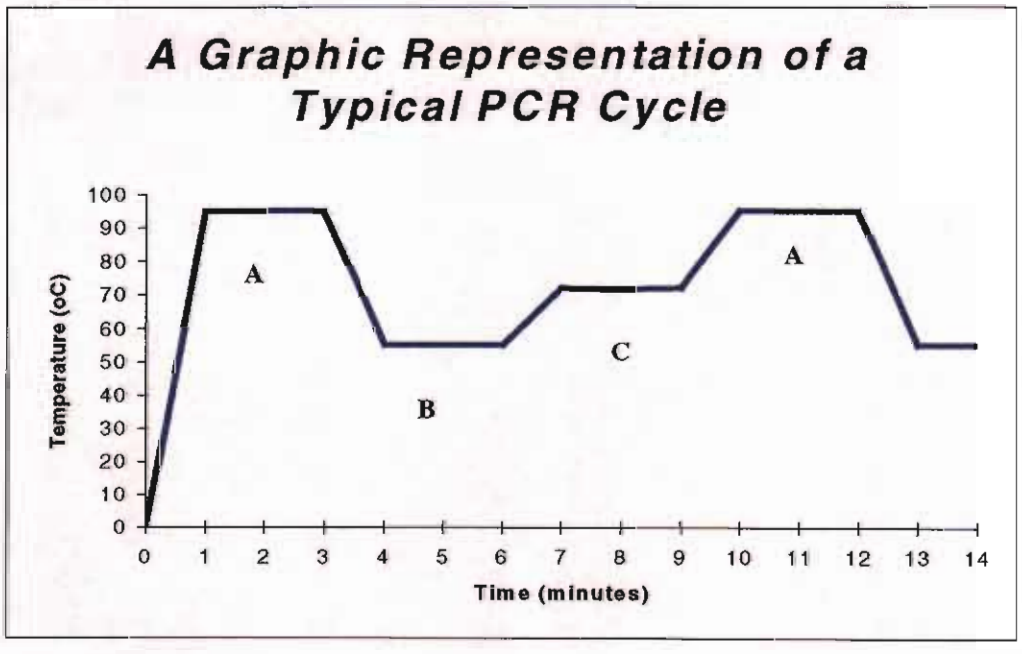
Figure 9: *A Schematic Diagram of the PCR Reaction.*

(http:www.//ornl.gov/hgmis/publicat/primer/pcr.html)

Primers (synthetic sequence specific oligonucleotides) should flank the gene of interest to allow selective amplification. DNA is synthesized by extension of the primer. The product of the PCR cycle is then capable of becoming template DNA for subsequent cycles of PCR. Target DNA (Bej *et al.,* 1991) is normally provided in the range 0.05-0.1 µg. The optimum template concentration is determined empirically for each protocol. In theory, only one intact strand of DNA is required for successful amplification.

PCR is carried out in the laboratory using a heat-programmable thermocycler. A thermocycler repeatedly and accurately changes the temperature of the reaction tubes, for a specific number of cycles. A typical temperature profile showing different heating and cooling phases, A B & C, is indicated in *Figure 10.*

Figure 10: *A graphical representation of a Typical PCR Cycle. Double Stranded DNA is denatured at 95°C. Primer Annealing occurs at sequence-dependent temperatures (typically 53°-68°C). DNA extension takes place at 72°C*



The thermal profile shown in *Figure 10* can be explained briefly as follows:

A. Double stranded DNA is denatured by heating to temperatures typically between 93 and 95°C for 5 minutes for the first cycle, and for about 1 minute during each subsequent cycle.

B. Annealing of the primers to the single stranded DNA usually occurs between 53°C and 68°C. The annealing temperature should be as high as possible to prevent non-specific hybridisation of the probe to the DNA template, and therefore, non-specific amplification. Annealing temperatures are usually dependent on the melting temperature of the primer. Primers with higher G and C content require a higher annealing temperature.

C. Extension is normally carried out at 72°C, which is the optimum temperature for the most common DNA polymerase used - *Taq* DNA polymerase. The polymerase synthesizes new complementary DNA strands from the site of the primer in a 5' – 3' direction.

Cycle number depends on the concentration of the template DNA – the cycle number is increased if the concentration is low, or of poor quality. PCR results in an exponential increase in the number of copies of template DNA present.

In addition to primers, template DNA and *Taq* DNA polymerase, PCR has several other requirements, which are briefly summarised as follows:

Magnesium ions: Mg^{2+} is essential for the activity of *Taq* DNA polymerase, and the concentration is optimised for each PCR protocol (usually between 1.5-4 mM).

dNTPs: dATP, dCTP, dGTP and dTTP should be added to the reaction mixture in equal concentrations – usually about 200 μM of each.

There is a stoichiometric relationship between dNTP concentration and Mg^{2+} concentration. At high concentrations, dNTPs and primers bind to magnesium and reduce available magnesium for *Taq* DNA polymerase. It is therefore necessary to establish the appropriate Mg^{2+} concentration for each reaction. For a more complete review of this subject see: M^{C}Pherson *et al.,* (1991); Ausbel *et al.,* (1995).

1.7.1 Detection of NPT II using PCR

The development of PCR has allowed rapid and accurate detection of foreign DNA from a variety of sources. PCR allows the detection of very low quantities of DNA, as theoretically, only one intact copy of the gene is required for successful detection. Furthermore, PCR is rapid, sensitive, and does not require any radioactivity or labelling steps.

Blake *et al.,* (1991) used PCR to screen for genetically modified alfalfa by targeting the insertion of both NPT II and β-glucuronidase (GUS) marker genes. A 785 bp fragment of the NPT II gene and a 1.09 Kb fragment of the GUS gene were amplified and the results verified by Southern blotting. The object of the experiment was to screen for the simultaneous integration of both genes into a plant genome.

Hamill *et al.,* (1991) transformed root cells of two strains of tobacco with both NPT II and GUS genes using *Agrobacterium tumefaciens*. These newly transformed plants were then screened for the inserted genes using PCR. A 700 bp fragment of the NPT II gene was amplified and a 1.2kb fragment of the GUS gene.

Padegimas *et al.,* (1993) used PCR to screen newly transformed transgenic tobacco and potato plants. The vector used for transformation, pBIN19, contained both NPT II from transposon Tn5 and the NPT III gene from *Streptococcus. Agrobacterium* infection may still persist in the new transformant for some weeks therefore the presence of both genes in the plant would indicate residual *Agrobacterium* infection. The presence of NPT II alone would indicate successful transformation. The primers used produced a 173 bp fragment of the NPT II gene and a 340 bp fragment of the NPT III gene.

Meyer (1995) developed two PCR protocols to detect genes inserted into the FLAVR SAVR ™ tomato. The first amplified a 172 bp fragment of the NPT II gene, and the second amplified a 427 bp sequence that connects the CaMV promoter to the anti-

polygalacturonase gene. These two genes were found to be unique to the FLAVR SAVR™ tomato when compared to non-GM tomatoes.

Towards the beginning of the present study Greiner *et al.*, (1997) published the first account of attempts to detect evidence of genetic engineering in highly processed foods. Tomato ketchup, pizza tomatoes and soup were investigated. A PCR protocol was developed to detect a 254 bp sequence of the anti-polygalacturonase gene in GM tomatoes. Good quality template DNA could be extracted from tinned pizza tomatoes and tinned peeled tomatoes, resulting in a confirmed GM-test. However, no GM-associated DNA could be detected in tomato soup. Neither could a PCR product be detected when using DNA extracts from tomato ketchup or tomato paste.

Also, towards the end of the work presented in this thesis, Parkes (1999) reported a general testing method to detect genetically modified plants. This method detected the promoter, terminator and antibiotic resistance genes inserted into plants. However, due to the fact that there are several of each in common use, and due to a widening variety becoming available, it is necessary to constantly update the methods. Using this broad approach genetically modified chicory, maize, oilseed rape, potato, papaya, soybean, squash and tomato are identifiable. This worker pointed out that detection of genes in tomato paste was again particularly difficult.

1.7.2 Problems Associated with PCR and Processed Foods

Cooked and processed foods present a particular problem for PCR. DNA can be degraded by both the manufacturing and cooking processes.

Degradation can be caused by the following (Parkes, 1999):

- Prolonged heat treatment, causing DNA hydrolysis – canned foods
- Chemical modification and DNA hydrolysis at low pH – tomato paste and similar tomato products
- Enzymatic degradation of DNA by nucleases – fresh foods

Another complicating factor is that plant DNA often co-extracts with molecules that can interfere with the PCR reaction. The problem can often be overcome by diluting the DNA sample. However, if the concentration of DNA in the sample is already low, this is not possible. Nested PCR may be used in samples where DNA concentration is too low to obtain a product by conventional PCR methods (Parkes, 1999). In the case of other genetically modified food products, such as refined oils, DNA detection methods are not applicable as no DNA is present after processing.

Common inhibitors of the PCR reaction in processed foods include the following:

- Cations e.g. Ca^{2+}, Fe^{3+}
- Trace heavy metals
- Carbohydrates
- Tannins, phenolics
- Salts and Nitrites

1.8 Detection of Genetically Modified Soy

Genetically modified soy was developed by the US biotechnology company, Monsanto. This plant has been transformed by the insertion of the 5-*enol*-Pyruvylshikimic Acid -3-Phospatase synthase (EPSPS) gene, isolated from the CP4 strain of *A. tumefaciens*. The EPSPS gene confers resistance to the herbicide glyphosate, whose Trademark is 'Round Up™' on the transgenic plant. Glyphosate (N-phosphonomethyl-glycine) is a broad-spectrum herbicide effective against most grasses and weeds, but yet, having minimal toxicity, limited persistence and no leakage into the water table (Padgette *et al.,* 1995).

Soy is a common ingredient in processed foods, and the bulk of soy consumed in the EU is imported from the US (where over 30% of the crop last year was genetically modified). In order to conform with EU legal requirements, it is necessary to have methods to detect modified food(s). There are several possible target genes for detecting genetically

modified soy – the promoter sequence (CaMV), the trait gene (EPSPS) or the terminator gene (P-nos).

Meyer *et al.,* (1996) developed a method to detect soy in processed meat products. Samples used in the study included soy protein concentrates, legumes, and meat products containing soy protein. A direct PCR assay was developed for use on concentrated soy products and this was combined with a nested PCR protocol designed to detect soy in processed meat products where the expected concentration of soy would be relatively low. The target sequence was the lecithin gene *Le1*. The results were verified by restriction digest and ELISA, using a commercially available kit. The results from the PCR protocol and ELISA assays were in agreement. This protocol was later extended by Meyer and Jaccaud (1997) to include primers for the promoter sequence and the EPSPS gene. This produced a very specific test for genetically modified soy.

Zimmermann *et al.,* (1998) evaluated nine different methods of extracting DNA from genetically modified soy samples. The DNA extracts were quantified by UV spectrophotometry, while the quality of the DNA was assessed using PCR. It was found that commercially available spin columns produced low amounts of high quality DNA, while simpler and more rapid methods of DNA extraction produced more, low quality DNA.

1.9 Experimental Objectives

Prior to the present study very few attempts had been made to develop methods for the detection of GM material in processed foods. It was clear that such methods could not be based on expressed protein since proteins in processed foods are likely to be denatured. The PCR methodology for detection of transgene DNA offered significant potential as a solution to this problem.

This study focused on the following aims:

1. Optimisation of DNA extraction protocols
2. Primer design
3. PCR protocol design

Chapter 2: Materials and Methods

2.1 Materials & Equipment

2.1.1 Chemicals

All chemicals used in this work were analar grade. Buffer salts and reagents were purchased from the Sigma, (Poole, UK) and stored at room temperature. *Taq* DNA polymerase, primers and PCR reaction components were purchased from Promega (Madison, WI) or Boehringer Mannheim (IN, USA) and stored at –20 ^{0}C until use. All water used for preparation of solutions was distilled and autoclaved.

2.1.2 DNA Purification

On occasion, DNA was further purified using commercially available kits for this purpose. The Wizard Genomic DNA extraction kit from Promega was used in an attempt to isolate genomic DNA from tomato paste. PCR amplified products were prepared for restriction digestion by purification using the QIAquick Purification kit from QIAGEN.

2.1.3 Molecular Weight Markers

The molecular weight markers used for electrophoresis were obtained from either Boehringer Mannheim or Promega. The designation of the markers was as follows:

- Promega PCR markers: these consisted of a ladder of 6 DNA fragments of the following sizes; 50, 150, 300, 500, 750 and 1000 base pairs.
- Boehringer Mannheim Molecular Weight Markers III: these consisted of a ladder of 13 DNA fragments derived from a *HindIII/EcoRI* digest of λ-DNA of the following sizes: 125, 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973, 5148, 21226 base pairs.
- Boehringer Mannheim Molecular Weight Markers V: these consisted of a ladder of 22 DNA fragments derived from a *HaeIII* digest of pBR322 of the following sizes; 8, 11, 18, 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540, 587 base pairs. On occasions these sets of markers were used in combination by mixing them 1:1 before loading on the gel.

2.1.4 Instruments and Equipment

All PCR reactions were carried out on a Hybaid (Ashford, UK) 'Express' Thermocycler. Electrophoresis was carried out using a 'Fast Track' mini gel box electrophoresis system and a Fisons (MA, USA) FEC 570 power pack. Gels were photographed using a Polaroid (MA, USA) Gel Cam with a UVi Tec Transilluminator. Spectrophotometric measurements of DNA quantity were made using a Spectronic 101 UV

spectrophotometer. The microwave used for DNA extraction was a Phillips ' Space cube' with a power output of 730W.

2.1.5 Origin of GM food samples

Genetically modified tomato puree was purchased from J Sainsbury Ltd (UK). Non-modified tomato puree was also purchased commercially. Soy standards containing from 0 to 2% GM soy were purchased from Fluka Biochemicals (MW, USA).

2.1.6 Computer Applications

The software and databases used during this work are detailed below:

- The SEQUENCHER program (Gene Codes Corporation, Ann Arbour, MI) was used to analyse DNA fragments for restriction sites.
- The DNA sequences of many of the genes present in GM foods may be obtained from GenBank. This database may be searched for sequence similarity using the BLAST family of algorithims.
- The software used to design PCR primers was obtained via the internet from the Whitehead Institute for genome research (Rozen and Skaletsky, 1998). The primer design software called Primer3 may be accessed at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

2.2 DNA Extraction Methods

During the course of this work, a variety of methods for extraction of genomic DNA were tested in an effort to obtain good quality template DNA, free from inhibiting components. These are described below. Buffers used in these extraction protocols are described in detail in *Appendix 1*.

2.2.1 Method A

DNA was extracted from tomato paste following the method of Greiner *et al.*, (1997). One gram tomato paste sample was weighed directly into an eppendorf tube. One millilitre of extraction buffer A (100 mM Tris-HCl [pH 8.0] containing 1 M NaCl, 20 mM EDTA, 2% w/v SDS – 500 µg/ml of Proteinase K was added and the samples were incubated at 60°C for 1 hour. The sample was then centrifuged at 9,000 rpm for 12 minutes. Six hundred micromillilitres of the upper aqueous phase was removed to a fresh tube and 1 volume chloroform:phenol:2-pentanol (25:24:1) was then added. After centrifuging at 9,000 rpm for 6 minutes the upper phase was again removed to a fresh tube. One volume of chloroform:2-pentanol (24:1) was added. The sample was centrifuged for 6 minutes at 9,000 rpm. The aqueous phase was again removed to a new tube and 0.1 volumes 3 M sodium acetate [pH 5] and 2 volumes 98% ethanol were added. The samples were incubated at –18°C for 2 hours. The precipitate was pelleted by centrifuging at 9,000 rpm for 12 minutes at 4°C, washed once with ice-cold 70% ethanol and air-dried. Finally the pellet was resuspended in 100 µl autoclaved distilled water. The extracts were stored in the short term at 4°C or in the long term at –20°C. In a

modification of this method the tomato puree was lyophilised and/or pulverised under liquid nitrogen (-196°C) using a mortar and pestle. Three hundred milligrams of the puree was suspended in 1 ml of extraction buffer A and an extraction carried out as above.

2.2.2 Method B

In this case, DNA was extracted by boiling, using the method of Lench, 1988. Three hundred milligrams of tomato puree sample was suspended in autoclaved distilled water or CTAB buffer (*Appendix 1*) and boiled for 15 minutes. The sample was then centrifuged at 9,000 rpm for 12 minutes. The supernatant could then be used directly for PCR, or further purified using a phenol/chloroform extraction (as outlined previously in **Method A**).

2.2.3 Method C

The CTAB extraction was performed following Ausbel (1995). 2-Mercaptoethanol (ME) was added to a CTAB Extraction Solution (*Appendix 1*) to a final concentration of 2% (v/v) and heated to 65°C. The tomato paste samples were pulverised under liquid nitrogen. The warm ME/CTAB solution was added to the tissue (1 ml per 100 mg), vortexed, and incubated for 1 hour at 65°C. One volume of 24:1 chloroform/isoamylalcohol was added to the tube, mixed by inversion and centrifuged for 5 minutes at 9,000 rpm. One-tenth volume CTAB/NaCl Solution at 65°C was added and the chloroform/isoamylalcohol extraction repeated. One volume CTAB Precipitation

Solution was then added and mixed by inversion. The tube was incubated at 65°C for 30 minutes. After centrifugation for 5 minutes at 3,000 rpm the supernatant was removed and the pellet resuspended in 100 µl high-salt TE buffer. The DNA was precipitated by adding 0.6 volumes of isopropanol, mixing by inversion and centrifuging at 9,000 rpm for 15 minutes. Finally the pellet was washed with 80% ethanol and resuspended in TE buffer.

2.2.4 Method D

A commercial extraction kit (Wizard Genomic DNA Extraction Kit, Promega) was assessed for its ability to provide high-quality DNA templates suitable for PCR. Tomato puree was frozen with liquid nitrogen and ground into a powder, as before. Forty milligrams of the frozen tissue was weighed into an eppendorf tube and 600 µl of Nuclei Lysis Solution (supplied with kit) was added and the tube vortexed. The tube was incubated at 65°C for 15 minutes. Three micromillilitres of RNase solution was added to the cell lysate and mixed by inversion. The tube was incubated at 37°C for 15 minutes. The sample was then cooled to room temperature before proceeding. Two hundred microliters of Protein Precipitation solution (supplied with kit) was added and the tube vortexed for 20 seconds. The tube was then centrifuged at 9,000 rpm for 6 minutes (precipitated proteins form a tight pellet) and the supernatant was removed to a fresh tube containing 600 µl isopropanol at room temperature for mixing by inversion. The tubes were centrifuged for 2 minutes at 9,000 rpm, and the supernatant was decanted off. 600 µl of 70% ethanol at room temperature was added and the tubes inverted several times to

wash the DNA. The tubes were again centrifuged for 2 minutes at 9,000 rpm. The ethanol was pipetted off and the pellet was air dried before adding 100 µl DNA Rehydration Solution (supplied with kit) and incubated overnight at 4°C. The DNA was also stored at this temperature.

2.2.5 Method E

One hundred milligrams of tomato paste was resuspended in 300 µl TE buffer. This was then microwaved at full power of 730 W (*Appendix 1*) for 1.5 minutes and centrifuged for 2 minutes at 14,000 rpm. The supernatant was then diluted using a range from 10^{-1} to 10^{-6} to reduce the concentration of inhibiting substances in the extract before PCR.

2.3 Quantitation of DNA

DNA was quantified by determining the absorbance at 260nm (A_{260}). A_{260} readings are quantitative for relatively pure DNA extracts in microgram quantities. Proteins absorb at 280nm so A_{260}/A_{280} ratio of the extract gives an indication of sample purity. A ratio of 1.8 indicates a pure extract. (Ausbel, 1995)

DNA was also visually quantified using agarose gel electrophoresis. The DNA extract was run on a 1.5% agarose gel with 600 ng of an appropriate molecular weight marker. The DNA was then quantified by visual comparison with a standard of known concentration.

2.4 Polymerase Chain Reaction

PCR was performed in a Hybaid 'Express' Thermocycler. This thermocycler used a 96 well heating block and had a heated lid obviating the need for an oil overlay. Positive and negative controls were performed with each reaction. The positive control used was the pVDH 394 plasmid supplied by Professor P. Dix (NUI Maynooth, Ireland). This plasmid contains both the 35S CaMV gene and the NPT II gene. A detailed map of this construct is given in *Appendix 2*. All oligonucleotide primers were synthesised by Genosys Biotechnologies Ltd (Cambridge, UK).

Table 3: A typical mixture in a PCR reaction used to detect GM foods.

Reagent	*Volume (µl)*
$MgCl_2$, 25 mM Solution	3
Thermophilic DNA Polymerase 10 X Buffer*, $MgCl_2$ - Free	5
PCR Nucleotide Mix, 10 mM	1
Upstream Primer, 1X stock 1 µmole/L	1
Downstream Primer, 1X stock 1 µmole/L	1
Taq DNA Polymerase, 5 U/µl	0.25
DNA Extract	1
Distilled Water	37.75
Final Volume	50

*(This buffer was 100 mM Tris-HCl [pH 9.0], 500 mM KCl, and 1% Triton X-100).

Typical reaction components for PCR amplification are shown in *Table 3*. To set up the reaction the components were added to a 0.5 ml thin walled PCR tube in the order shown below. It was important to add the $MgCl_2$ *before* the *Taq* DNA polymerase and the DNA extract was always added last. Tubes were thoroughly mixed before placing in the thermocycler's heating block.

Having placed the PCR mixture in the thermocycler it was necessary to carry out repeated cycles of heating and cooling in order to get amplification of the target sequence. A typical temperature profile used for PCR is given in *Table 4*. This profile was used for the amplification of the NPT II gene segment using primers NPT1 and NPT2 as described in Section 3.1.3. The same profile, with minor variation, was used for amplification of the CaMV gene of GM soy (Section 3.2.2 of Results).

Table 4: The thermocycler program used in the detection of genetically modified tomato.

Step	Temperature (^{0}C)	Time (min)	Number of Cycles
(1)			
Initial Denaturation	95	2	1
(2)			
Denaturation	95	0.5	
Annealing	55	1	30
Extension	72	1	
(3)			
Final Extension	72	5	1
(4)			
Soak	4	indefinite	1

Having amplified the DNA sequence of interest a 10 µl PCR reaction mix was mixed with 2 µl of bromophenol blue loading dye and electrophoresed through a conventional 1% agarose gel, then stained with ethidium bromide and viewed under UV light (see Section 2.6). The amplified product was visualised as a band at the expected molecular weight.

2.5 Restriction Digest of PCR Products

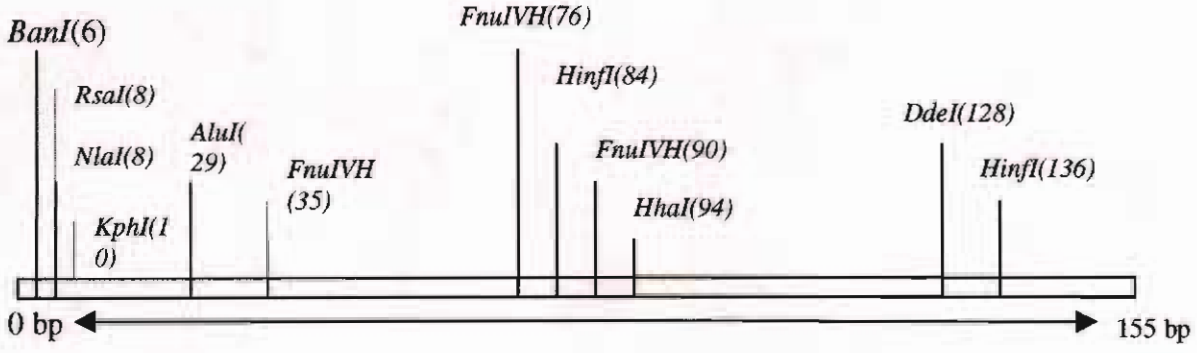
To verify that the PCR reaction amplified the correct sequence, a restriction digest of the amplified product was carried out. PCR products were purified using the QIAquick Purification Kit (Qiagen, Crawley, UK). A computer program called 'SEQUENCHER' (Gene Codes Corporation, Ann Arbour, MI) was used to determine the most suitable restriction enzyme for the digest using the sequence of the fragment produced by PCR. *Figure 11* shows a detailed restriction map of the relevant part of the NPT II gene used in the detection of GM tomato paste (see Section 3.1.2). It was found that *Hha1* targeted the DNA fragment at position 94 producing two fragments 61 bp and 94 bp in size. The restriction endonuclease *Hha1* was obtained from Promega.

Hha1 (Promega, Madison, WI) has the following Recognition sequence:

5' ..GCG↓C.. 3'

3' ..C↑GCG.. 5'

***Figure11**: Restriction map of a 155 bp sequence of the NPT II gene*



The restriction digest was set up as follows and incubated for 3 hours at 37°C.

PCR product	15 µl
10x buffer *	2 µl
Hha1 enzyme **	0.5 µl
Sterile Water	2.5 µl

*The buffer was 100 mM Tris-HCl [pH 7.9], 500 mM NaCl, 100 mM $MgCl_2$ and 10 mM DTT.

**The enzyme concentration was 10 U/µl.

2.6 Conventional Agarose Gel Electrophoresis

After PCR, the DNA bands were visualized by agarose gel electrophoresis. A 1% agarose gel was prepared by dissolving agarose with heating (Promega) in TAE buffer (*Appendix 1*) and allowing it to set upon cooling. Gels were run using a Hybaid 'Fast track' mini gel electrophoresis system. Ten micromillilitres of PCR product were mixed with 2 µl bromophenol blue loading dye and loaded into wells on the gel. The gel was run at 90 V for 45 minutes in TAE buffer, and subsequently soaked in a 5 µg/µl solution of ethidium bromide (10 mg/ml). Promega PCR Markers or Boehringer Mannheim molecular weight markers V and III (combined in equal volumes) allowed sizing of PCR products (Section 2.1.3). The gel was then photographed under UV light using a UVi Tec Transilluminator and a Polaroid Gel Cam (Kodak) Camera in conjunction with an orange filter.

2.7 Dot Blot

A "dot blot" is a procedure that involves generation of a digoxygenin (DIG) labelled DNA probe complementary to a region of the gene to be detected. Such probes can be easily generated by PCR in the presence of DIG-dUTP. The probe is then hybridised with the gene and detected using anti-DIG antibodies with the alkaline peroxidase (AP) enzyme attached. This method overall is less sensitive than PCR, but has the advantage of not being sensitive to PCR inhibitors. For these studies the DIG High Prime labelling and Detection Starter Kit I from Boehringer Mannheim was used.

2.7.1 Construction of DIG Labelled Probes

DIG-11-dUTP (Boehringer Mannheim) was added to the PCR reaction in place of dTTP becoming incorporated into the PCR product. PCR was performed as above with 0.5 nmol or 1.0 nmol DIG-11-dUTP added to the reaction mixture.

2.7.2 Immobilisation of DNA on to a Positively Charged Nylon Membrane

An appropriate nylon membrane (Boehringer Mannheim) was cut to size, placed on the surface of distilled water and allowed to submerge for 10 minutes. The blotting manifold (Millipore, France) was washed with distilled water. A square piece of filter paper (Whatman) was cut to size and soaked in distilled water. The manifold was then assembled, ensuring that it was airtight and free of bubbles. 1 M NaOH and 200 mM EDTA was added to each DNA sample to give a final concentration of 0.4 M NaOH and 10 mM EDTA. The samples were then denatured in a boiling waterbath for 10 minutes and microcentrifuged at 14,000 rpm for 5 seconds. The membrane was pre-washed with 500 µl distilled water. The samples were then applied to the membrane, and each well was rinsed with 500 µl 0.4 M NaOH. The membrane was removed from the manifold, rinsed well with 2X SSC (*Appendix 1*) and allowed to air-dry. The DNA was immobilised on the membrane by crosslinking on a transilluminator for 5 minutes. The membrane was then stored at room temperature.

2.7.3 Hybridisation with DIG-Labelled Probe

The membrane was prehybridised in a sealed plastic bag with 20 ml of prehybridisation buffer (*Appendix 1*) per 100 cm^2 membrane at 68°C for at least 1 hour. The membrane was agitated occasionally. The prehybridisation buffer was replaced with hybridisation solution containing denatured DIG-labelled probe DNA (2.5 cm^3 hybridisation solution per 100 cm^3 membrane) and incubated at 68°C overnight. The membrane was then washed 2 x 5 minutes with 2X SS containing 0.1% SDS at room temperature and 2 x 15 minutes in 0.1X SSC containing 0.1% SDS at 68°C.

2.7.4 Immunological Detection of DIG-Labelled DNA

The membrane was rinsed for 5 minutes in buffer 1 (for all buffers, see *Appendix 1*) and incubated at room temperature for 30 minutes in buffer 2. Four micromillilitres anti-DIG-AP was diluted in 20 ml buffer 1 and incubated with the membrane for 30 minutes. The membrane was washed twice in buffer 1 and equilibrated for 5 minutes in buffer 3. The membrane was then incubated in 10 ml of freshly prepared colour solution (200 µl colour solution in 10 ml of buffer 3) sealed in a plastic bag overnight, at room temperature. Incubating the membrane in buffer 4, for 5 minutes stopped the reaction. The membrane was then photographed to maintain a permanent record.

Chapter 3: Results

3.1 Introduction

At the outset of this project several different methods for the detection of Genetically Modified foods, together with their limitations, were highlighted. As discussed in Section 1.7, the most reliable method appeared to be detection of the foreign, inserted DNA that was present in GM foods, but not in their unmodified counterparts. The exact construction of the gene cassette inserted in many GM foods is not in the public domain. However, companies *are* required to divulge the exact DNA sequence of the inserted DNA to regulatory authorities when registering novel foods, but again this information is not made available to the general public. However, some data is available from company patents, publications of regulatory affairs and environmental impact reports. It is known that the most commonly used promoters and terminators in commercially available transgenic plants are the CaMV 35S promoter and the nopaline synthase (*nos*) terminator (see Sections 1.3.1 and 1.3.2 of the literature review). The most commonly used marker gene is the neomycin phosphotransferase gene (NPT II).

In 1997, when this project commenced, there were officially no genetically modified foods in Ireland. No labelling of GM foods was required at the time. However, more than 90% of the soy meal imported into Ireland originated in the United States. The US does not distinguish between modified and unmodified soy: all soy beans are bulked after harvesting and processed together. At the time it was thought that up to 5% of the US soy meal entering Ireland might be genetically modified. This meal found its way into a vast range of prepared snack foods, animal feedstocks, and was added as a filler to sausages, puddings, pies and many other foodstuffs.

The only commercially available, 100% GM foodstuff available at the time of writing was the tinned GM tomato paste sold by J Sainsbury, UK. This was the first GM product to appear on British supermarket shelves. This product was derived from GM tomatoes initially developed by Zeneca Plant Science, and contained a truncated form of the polygalacturonase gene under the control of the CaMV promoter and the *nos* terminator. The gene cassette also contained the NPT II gene as a selectable marker. No fresh GM tomato fruit was available.

Due to the extensive processing involved in paste production, GM tomato paste would present serious analytical difficulties due to its low pH and extensive fragmentation of host DNA. The extreme heating used during processing would certainly denature proteins and would be expected to promote any non-specific reactions between tomato components and DNA.

3.1.1 Source of GM Materials

Throughout this project genetically modified food samples and standards proved difficult to source. The GM tomato paste used in these experiments was not available in Ireland. The only other means of obtaining GM material was in the form of genetically modified soy or maize standards (0 to 2% GM) which became available towards the end of the project, from Fluka Biochemicals Ltd (MW, USA). A 100% GM soy or maize standard could not be obtained. As a result of these constraints, initial studies focused on GM tomato paste. GM soy was tested later.

3.1.2 Design of PCR Primers for GM Tomato Paste

The inserted DNA in the tomato paste commercially available from J Sainsbury UK is described in Section 1.5. Several potential PCR targets could have been selected; the CaMV promoter sequence, the nos terminator or the NPT II marker gene (Section 1.5). None of these genes are normally present in plant genomes, but are often inserted into genetically modified plants. However, the issue of consumer concern (Section 1.1), over the use of antibiotic resistance genes led us to choose the NPT II gene as the PCR target. The truncated polygalacturonase gene in this construct could not be used as the PCR target since it is already present in plant tissues.

The DNA sequence of NPT II was obtained from GenBank (Accession Number: V00618). The software used to design PCR primers was obtained *via* the internet from the Whitehead Institute for genome research (Rozen and Skaletsky, 1998). The primer design software called "Primer3" may be obtained from the web site http://www-genome.wi.mit.edu/genome_software/other/primer3.html. In addition to inputting the source DNA sequence, the program allows the user to specify parameters such as primer size, primer melting temperature, GC-content of primer, product size and melting temperature. The program attempts to minimise primer-primer interaction and primer self-complementarity.

In this case we chose an optimum primer length of 20-mer, with GC content of 50% giving a product size of 155 bp. It was important that the amplified product size was below the average DNA length of tomato paste. The average length of tomato paste DNA was reported to be 400 bp (Ford *et al.,* 1996). It was necessary to have a product of suitable size to be easily detected by agarose gel electrophoresis. The program, "Primer3", selected the pair of primers shown in *Table 5* as most closely matching the input criteria.

Name	Primer Sequence	%GC	Tm
NPT1*(f)*	**5'-CGTTGGCTACCCGTGATATT –3'**	**50**	**63.5**
NPT2*(r)*	**5'-AGTAAAGCTTGGGGTCTCAG –3'**	**50**	**64**

Table 5: The Primer Sequence, %GC content and the melting temperature of the NPT1 NPT2 primers, designed to detect the NPT II gene. Note: the annealing temperatures for these primers were provided by the manufacturers and differ slightly to those calculated by the "Primer3" software. (f=forward, r=reverse)

These primers amplify the 155 bp DNA fragment of the NPT II gene ranging from position 815 to 969 bp of the DNA sequence (see *Figure 12*).

5'- cgttgg ctacccgtga tattgctgaa gagcttggcg gcgaatgggc tgaccgcttc ctcgtgcttt acggtatcgc cgctcccgat tcgcagcgca tcgccttcta tcgccttctt gacgagttct tctgagcggg actctggggt tcgaaatga –3'

Figure 12: Fragment of the NPT II gene amplified by PCR. The NPT1 *and* NPT2 *annealing sites are underlined, while the highlighted section is the* Hha1 *restriction site.*

3.1.3 Design of the Polymerase Chain Reaction Assay

The assay was developed using the NPT1*(f)* and NPT2*(r)* primers designed in section 3.1.2. The reaction mix contained the following components in a final volume of 50 µl:

	Volume (µl)
25 mM $MgCl_2$	3
PCR Buffer	5
10 mM PCR Nucleotide Mix	1
NPT1 1X stock (1µmole/l)	1
NPT2 1X stock (1µmole/l)	1
Taq DNA Polymerase (5 U/µl)	0.25
DNA Extract	1
Distilled Water	37.75

The cycling parameters were as follows: tubes were heated to 95°C for 2 minutes to denature the target DNA. The mix was then subjected to thirty cycles of the following temperature profile: 95°C for 30 seconds, 55°C for 1 minute to allow for primer annealing and 72°C for 1 minute to allow for extension. Finally, extension was performed for 5 minutes at 72°C whereupon the reaction mixture was cooled to 4°C and held at that temperature until electrophoresis could be carried out (Section 2.4). Electrophoresis was carried out on a 1% agarose gel to visualize DNA bands produced by PCR (Section 2.6).

The PCR assay method was tested using a plasmid containing the NPT II gene. Professor Philip Dix (NUI Maynooth), kindly supplied the pVDH 394 plasmid. This plasmid was used in the initial development of the PCR assay method and thereafter used as a positive control. A map of the pVDH 394 plasmid is shown in *Appendix 2.*

Figure 13 shows the results of a typical PCR assay where the 155 bp fragment of the NPT II gene was amplified. No optimisation was necessary on the temperature profile of the PCR reaction, dNTP concentration, primer concentration or $MgCl_2$ concentration because the parameters, as described in section 2.4, were observed to be successful. The method could amplify template DNA at an initial concentration of 15 ng (*Figure 13).*

All gels were run in the presence of appropriate controls. DNA was extracted from both unmodified and GM tomato paste for use in the PCR reaction - the unmodified tomato DNA extract was used as a control for non-specific binding throughout. Negative controls included sterile, distilled water in place of template DNA.

Having obtained a sensitive and reproducible assay method for the NPT II gene the next step was to attempt the detection of this gene in a "real" sample. As mentioned above GM tomato paste was the only commercially available food that could be used to test the assay method.

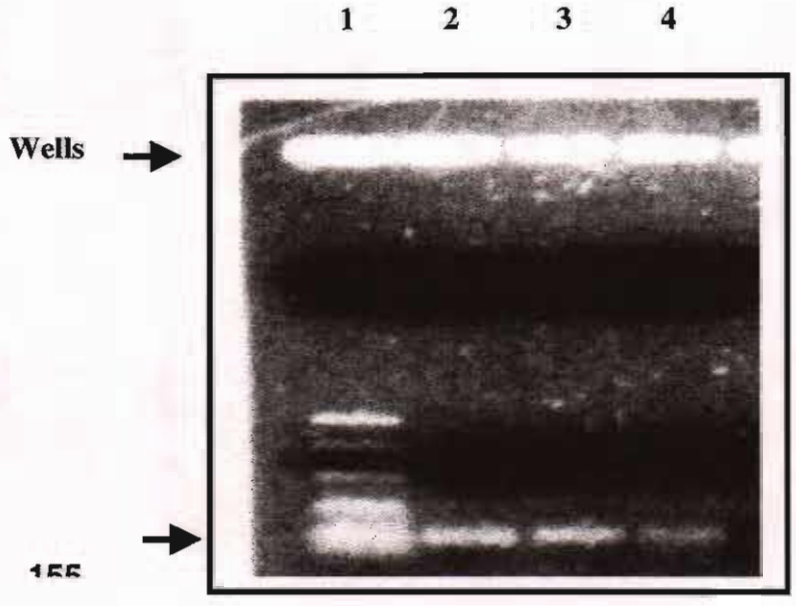


Figure 13: Lane 1 – Promega PCR Markers (50, 150, 300, 500, 750, 1000 bp). Lanes 2-4: amplified 155 bp DNA fragment of the NPT II gene from pVDH 394. Lane 2 - 1.5 μg plasmid DNA, Lane 3 – 0.15 μg plasmid DNA, Lane 4 – 15 ng plasmid DNA

3.1.4 Extension of the PCR Method to Tomato Paste

There are three distinct steps involved in the detection of genes in a food sample by PCR:

DNA extraction from the sample

Amplification – by PCR

Visualization – usually by agarose gel electrophoresis and ethidium bromide staining

The authenticity of the DNA fragment may then be verified by restriction digest or Southern blotting.

It was found that the most technically challenging and time-consuming step in the process was the extraction of DNA. Initial attempts at DNA extraction from tomato paste, used the method of Greiner *et al.*, (1997), who claimed successful extraction of DNA from tomato paste and other highly processed tomato products. Details of this method are described in Section 2.2.1 under Method A. Despite repeated attempts no PCR product was obtained using DNA extracted from tomato paste by this method. At the stage of phenol/chloroform extraction a heavy precipitate was observed. This material appeared to be protein as verified by a Biuret test (Sapan *et al.*, 1999). The protein was obviously not fully degraded by the proteinase K present in the extraction buffer. This protein may have interfered with the subsequent extraction steps. Alternatively, PCR inhibitors, likely to be present in tomato paste, may also have interfered with the assay.

Freeze drying of tomato paste samples was attempted in order to concentrate the paste such that a greater amount of DNA might be extracted. However, it was found that the samples were very difficult to lyophilise thoroughly. Even when, after freeze-drying, the samples were frozen using liquid nitrogen and pulverised with a mortar and pestle, no PCR product was obtained.

In the belief that the source material was yielding insufficient amounts of template DNA for PCR, the simplified DNA extraction protocol of Lench *et al.,* (1988) was attempted. This method involves boiling of the samples as described in Section 2.2.2. (**Method B**). Samples were either used for PCR immediately, or further extracted with phenol/chloroform. Again, this method was unsuccessful. The CTAB extraction method from Ausbel, (1995) was then attempted for tomato paste (see Section 2.2.4 for description). The Wizard™ Genomic DNA extraction kit (Promega) was also used to extract DNA from tomato paste (see Section 2.2.5). This kit uses spin columns to bind DNA from the sample which is subsequently eluted. It was found that the resulting DNA extracts contained high quantities of precipitated protein and pigments from the tomato paste.

For all of the extraction protocols it was found that some of the red pigments of tomato paste were carried over into the isolated DNA preparation. It was found that, while the fragment of the NPT II gene from the positive control pVDH 394 plasmid DNA was amplified, no amplification of any of the extracted DNA samples was observed, even when absorbance readings at 260 nm indicated the presence of DNA.

We were forced to conclude that either an inhibitor of the PCR reaction was being co-extracted with the DNA, or that the final extracts did not in fact contain any DNA. To check for the presence of DNA in the extracts they were subjected to electrophoresis. *Figure 14* shows an electrophoretogram of DNA extracts obtained by direct extraction. Also shown, for comparison, is the DNA extract obtained using the microwaving method that eventually proved successful. From this gel it is clear that the DNA extracts *had* DNA present, as judged by electrophoresis, yet failed to yield a PCR product. Therefore, it seemed likely that some inhibitor of the PCR reaction might be present in the extracted DNA.

To check for the presence of a PCR inhibitor, DNA extracts were prepared and spiked with 1µg pVDH 394 plasmid DNA. PCR was performed as usual and it was observed that while the positive control produced a band of the correct size, no amplification of the positive control DNA took place in the presence of the DNA extracts. This clearly showed that something *was* present in the DNA extracts that were inhibiting the PCR reaction. The source of the PCR inhibition could not be identified, but could possibly have been due to contamination with either lycopene or polyphenols (Gartner *et al.,* 1997; Paganga *et al.,* 1999) that are normally present in tomato fruit. If the inhibitor were reversible, it should be possible to overcome inhibition of this type by dilution of the DNA sample. The dilution would decrease the concentration of inhibitor in the PCR assay to a level where its effect on the *Taq* DNA polymerase might be negligible.

It was subsequently found that the influence of the PCR inhibitor *could* be ameliorated by dilution of the sample. With this knowledge, it proved possible to design a simplified protocol for the detection of the NPT II gene in tomato paste. The details of this method are described in Section 2.2.5. Briefly, 100 mg tomato paste sample was resuspended in 300 µl TE buffer (*Appendix 1*). It was microwaved for one minute and centrifuged at 14,000rpm for 5 minutes. The supernatant was then used directly for PCR, as described in Section 2.2.5. Any PCR inhibitors co-extracting with the DNA were diluted out before PCR was performed. *Figure 15* illustrates how this simplified protocol was successful in overcoming inhibition of the PCR assay. It can be seen from the gel that while the undiluted extract did not produce a PCR product, when serial dilutions to 10^{-6} were prepared and used for PCR, amplified DNA fragment bands were produced (*Figure 15*).

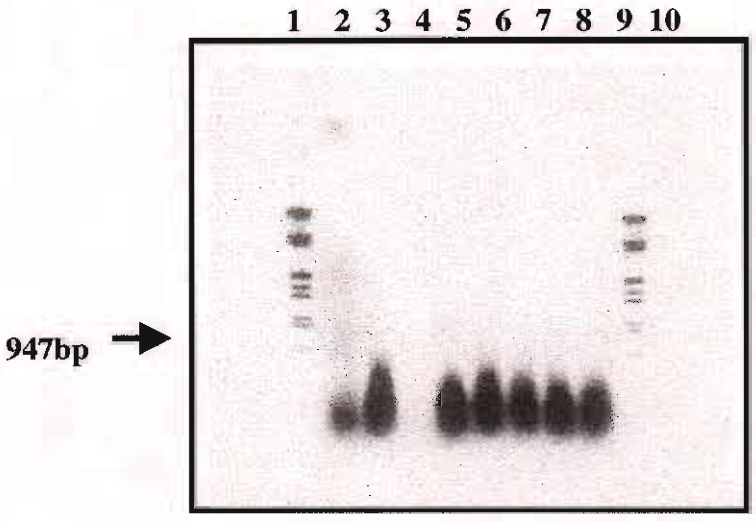


Figure 14: Lanes 1 & 10 Molecular Weight Marker III (Boehringer Mannheim). Lane 2 – DNA extracted from GM tomato paste by microwaving. Lanes 3-9 DNA extracted from GM tomato paste using phenol/chloroform extraction. The DNA extracts all show an average length of ~500 bp.

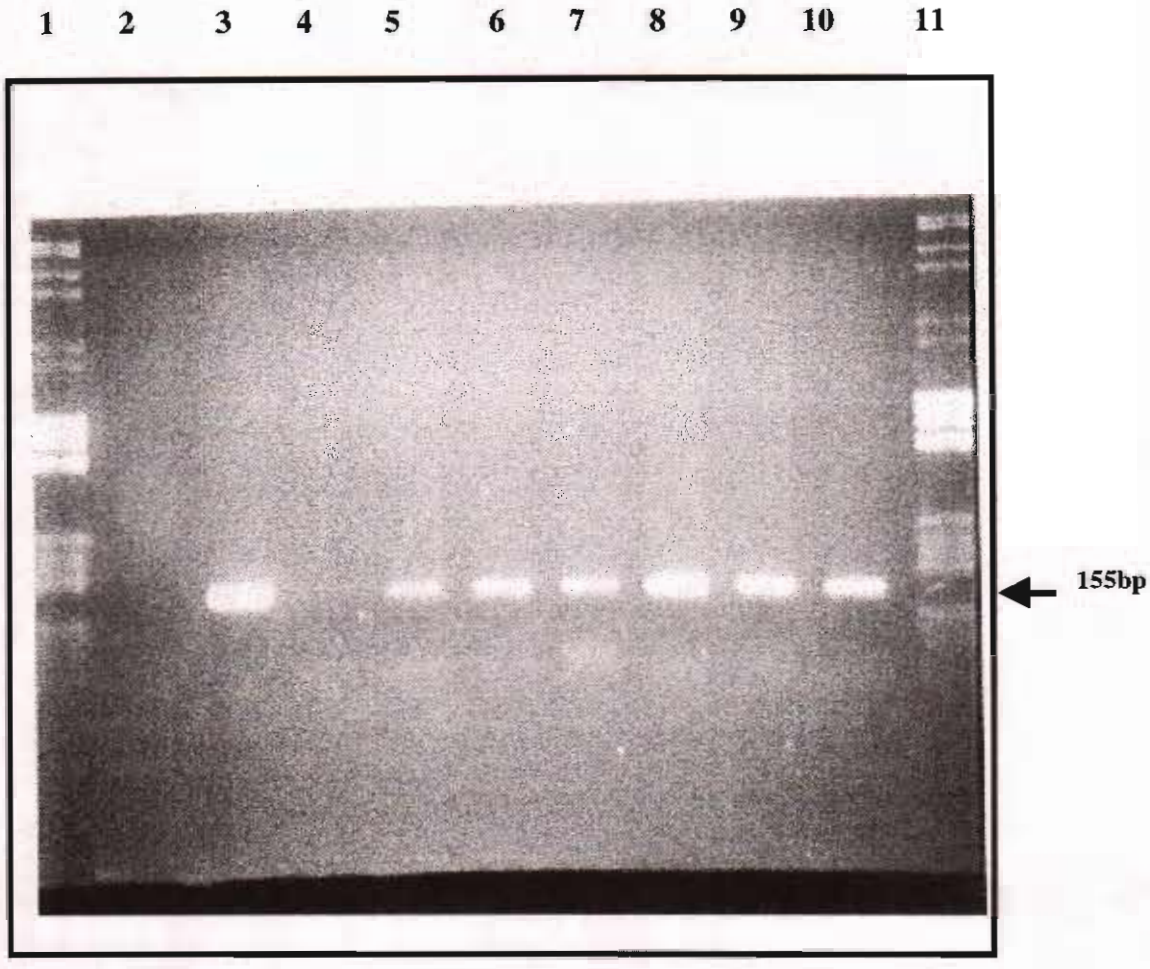


Figure 15: A 155 bp fragment of the NPT II gene amplified from tomato paste by PCR. Lanes 1 & 11– Combination of Molecular Weight Markers III and V (Boehringer-Mannheim). Lane 2–negative control (no template DNA), Lane 3-positive control, pVDH 394 plasmid DNA, Lane 4–undiluted GM tomato paste DNA extract, Lane 5–10^{-1} dilution, Lane 6–10^{-2} dilution, Lane 7-10^{-3} dilution, Lane 8-10^{-4} dilution, Lane 9-10^{-5} dilution, Lane 10-10^{-6} dilution.

3.1.5 Restriction Digest of PCR Product

To verify that the amplified PCR product was indeed derived from the NPT II gene, it was subjected to restriction digestion. A unique restriction site for the restriction enzyme *Hha1* was identified on the amplified PCR product (*See Figure 11*). This enzyme should cleave the PCR product into two fragments of 61 bp and 94 bp. The 155 bp product amplified from the NPT II gene by PCR was removed from the PCR reaction mixture using the QIAquick Purification Kit (Qiagen) (*Section 2.5*). The kit uses spin columns to bind the PCR product and so remove the residual components of the PCR reaction. A restriction digest was performed on the PCR product as per Section 2.5, using the enzyme *Hha1*. *Figure 16* shows that the PCR product *was* cleaved into the two expected DNA fragments. The presence of this restriction site at the correct position on the amplified fragment further authenticated the PCR product and indicates the specificity of the reaction.

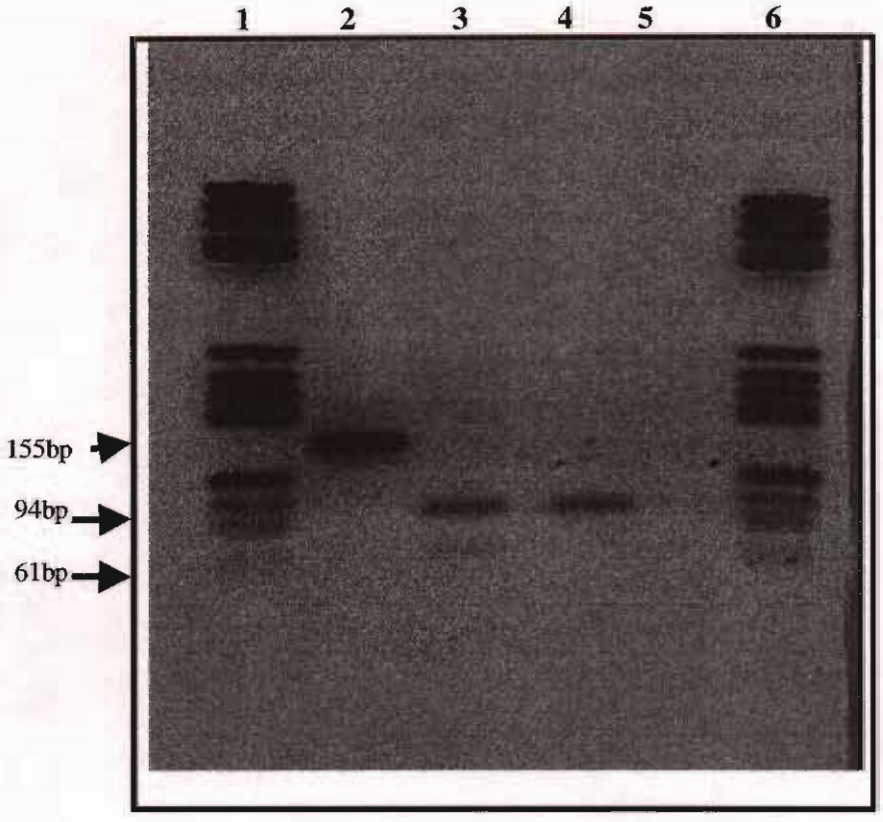


Figure 16: Restriction Digest, using Hha1, of the DNA bands produced by the PCR reaction. Lanes 1 & 6 –Molecular Weight Marker V (Boehringer-Mannheim) 2-Positive Control DNA amplified using PCR protocol, 3, 4 & 5 – Restriction Digests of PCR products

3.1.6 "Dot Blot"

To further simplify the detection of genetically modified tomato paste, an attempt was made to develop a "dot blot" assay for the NPT II gene. This involved the production of a digoxygenin labelled probe capable of binding to the NPT II gene. The detailed protocol for the generation of dig-labelled probes is given in Section 2.7. Briefly, amplification of the 155 bp segment of the NPT II gene was carried out in the presence of digoxygenin-labelled dUTP. The resulting amplified product was labelled with digoxygenin (*Figure 17).* Hybridization using the latter probe was then detected using an anti-digoxygenin antibody system. The dot-blot procedure is simple and robust, allows for a higher sample throughput, negates the need for gel electrophoresis and has potential for automation. However, the "dot blot" method is less sensitive than a PCR assay.

Figure 17 shows the production of DIG-labelled probes for the NPT II gene. Incorporation of the DIG label is observed as an upward bandshift in band mobility on the electrophoretogram.

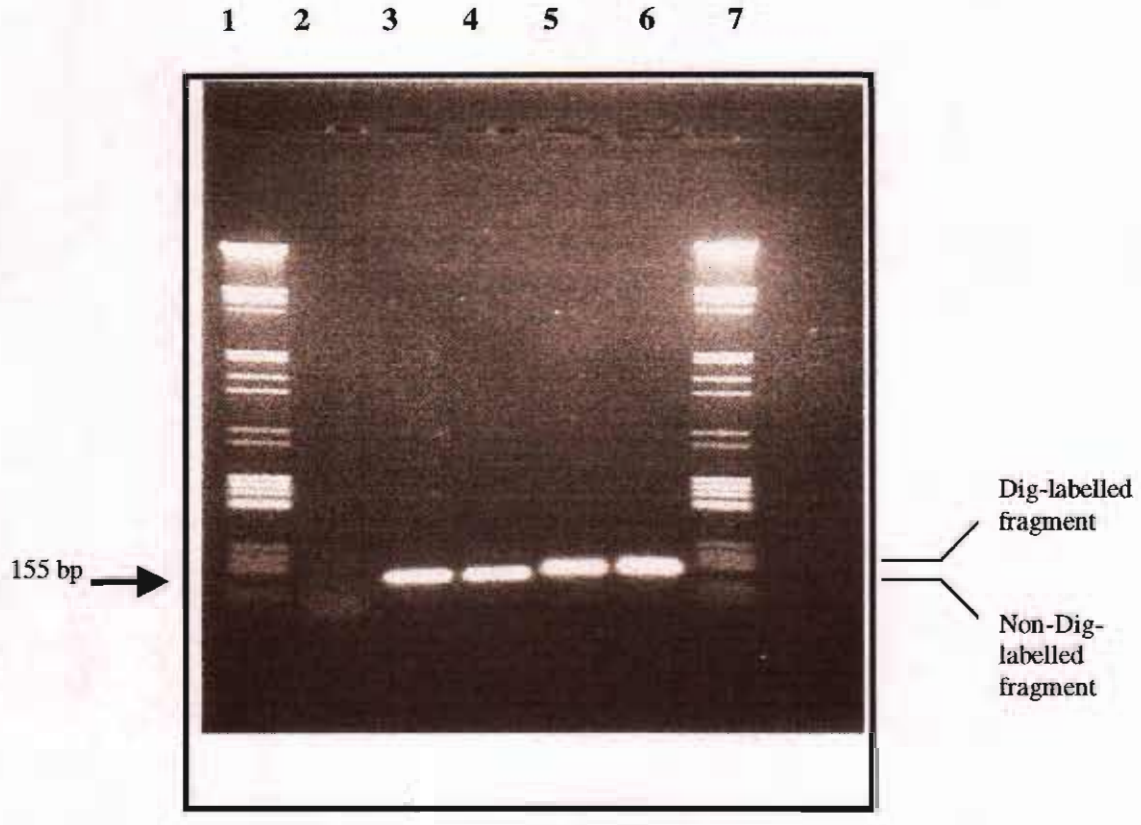


Figure 17: An agarose gel showing the production of DIG-labelled DNA probes for use in "Dot-Blot". Lanes 1&7 – Combination of Molecular Weight Markers III and V (Boehringer Mannheim), 2 – negative control (no template DNA). The shadow band is primers. 3 & 4–unlabelled PCR product, 5–Digoxygenin-labelled PCR product (0.5 nmol DIG), 6 -Digoxygenin-labelled PCR product (1.0 nmol DIG)

The dot blot was performed on the following samples and probed with the DIG-labelled probes as described in section 2.7.

- Positive control pVDH 394 plasmid DNA (both labelled and unlabelled)
- Undiluted and diluted GM tomato paste in a dilution range of 10^{-1} and 10^{-6}.
- GM tomato DNA extracted according to Method A, and used undiluted and diluted.
- GM tomato paste DNA extracted by microwaving, and used undiluted and diluted

The DIG-labelled probes were detected visually – a positive result showed a purple/pink dot on a white background. Results are only qualitative and limited to either indicating the presence or absence of the target DNA fragment in the sample. The positive control DNA produced the expected result (*Figure 18*, A1, B1 and C1). However, no colour change was observed for any of the DNA samples extracted from tomato paste. While these results prove that the assay is effective, in principle, there is still a problem with substances in tomato paste that interfere with access to the NPT II gene. This assay was not pursued further for the tomato paste but might prove useful for other GM foods or for tomato fruits.

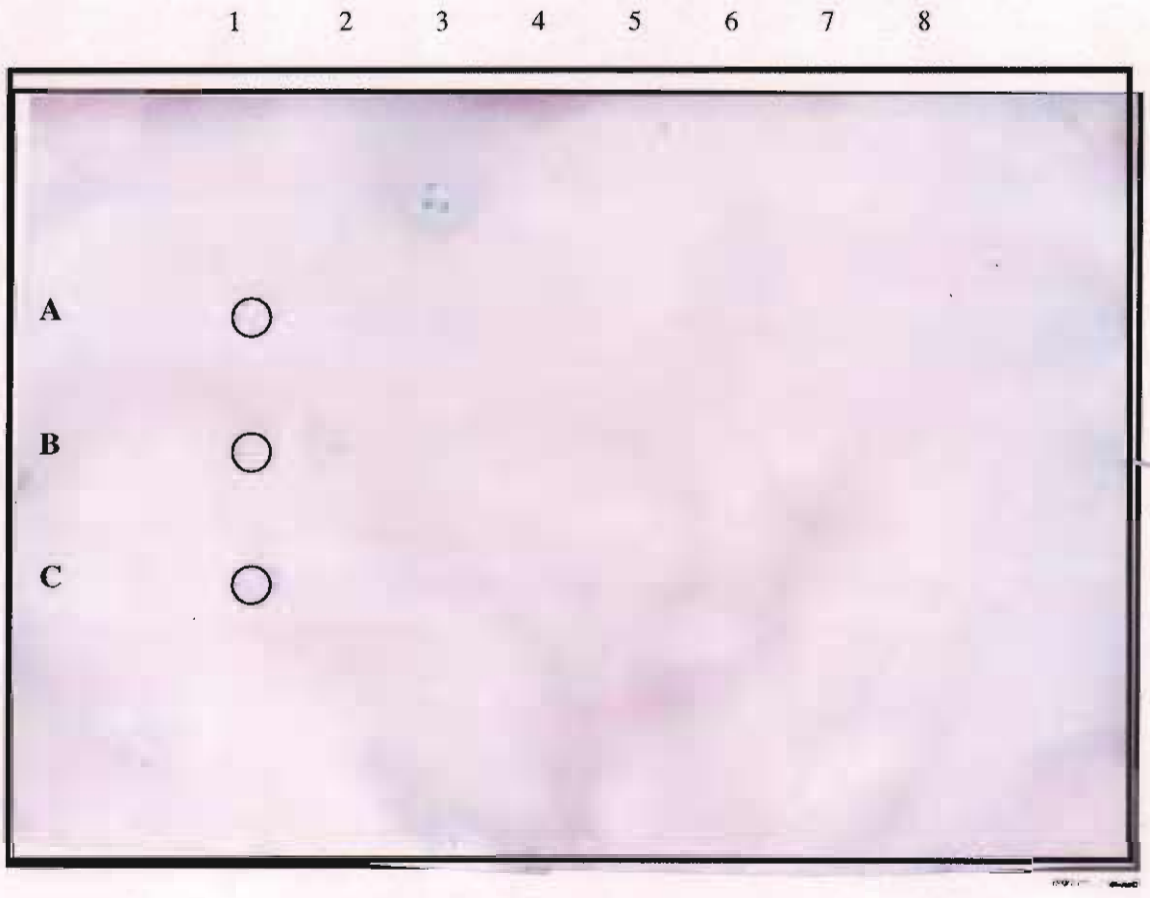


Figure 18: "Dot blot" of GM Tomato Paste as described above. A, B and C are positive controls containing the unlabelled pVDH 394 plasmid. All other samples and controls were negative. Note: reproduction of the diagram proved difficult and the positive results are circled.

3.2 Genetically Modified Soy

Genetically modified soy forms a high percentage of the annual US soy crop – about 50 million acres of GM soy were planted in the US in 1999. GM crops are not differentiated from non-GM crops in the US, which is the major supplier of soy to the EU. This results in unlabelled GM soy being incorporated into foodstuffs available within the EU.

3.2.1 Detection of Genetically Modified Soy

Genetically modified soy standards containing from 0% to 2% genetically modified soy by weight, are commercially available from Fluka Biochemicals Ltd. The standards are supplied in the form of freeze-dried, homogenised soy powder. The DNA is degraded during extensive processing, but is not as degraded when compared to tomato paste. A sample of 100% GM soy was not commercially available.

3.2.2 Choice of PCR Target

The CaMV 35S promoter was chosen as the PCR target to detect GM soy. This controlling element is present in a wide range of GM plants, allowing for a broadly applicable test method.

The gene sequence of CaMV 35S was obtained from GenBank (Accession number E01311). Primers were designed using the "Primer3" software as described for the NPT

II gene in Section 3.1.2. The primer pair in *Table 6* was used to amplify a 200 bp region of the CaMV gene.

Name	Primer Sequence	%GC	Tm
CaMV1	**5'-CTACAAATGCCATCATTGCG-3'**	**45**	**66.2**
CaMV2	**5'-AAGGATAGTGGGATTGTGCG-3'**	**50**	**68.3**

Table 6: The Primer Sequence, %GC content and the melting temperature of the CaMV1 CaMV2 primers, designed to detect the CaMV 35S promoter.

3.2.3 Design of the Polymerase Chain Reaction Assay

The assay was developed using the primers designed in section 3.2. The cycling parameters were as follows: 94°C for 5 minutes followed by thirty cycles of: 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. Finally, extension was performed for 5 minutes at 72°C and the reaction mixture was cooled to 4°C and held at that temperature until electrophoresis could be carried out. The reaction mixture used was identical to that used for detection of the NPT II gene (Section 3.1.3). The PCR protocol was applied to the pVDH 394 plasmid, which also contains the 35S CaMV promoter (acting as a positive control). It was found that the primers successfully amplified the 200 bp fragment of the gene (*Figure 19*). Positive and negative controls were included, as set out in Section 3.1.3.

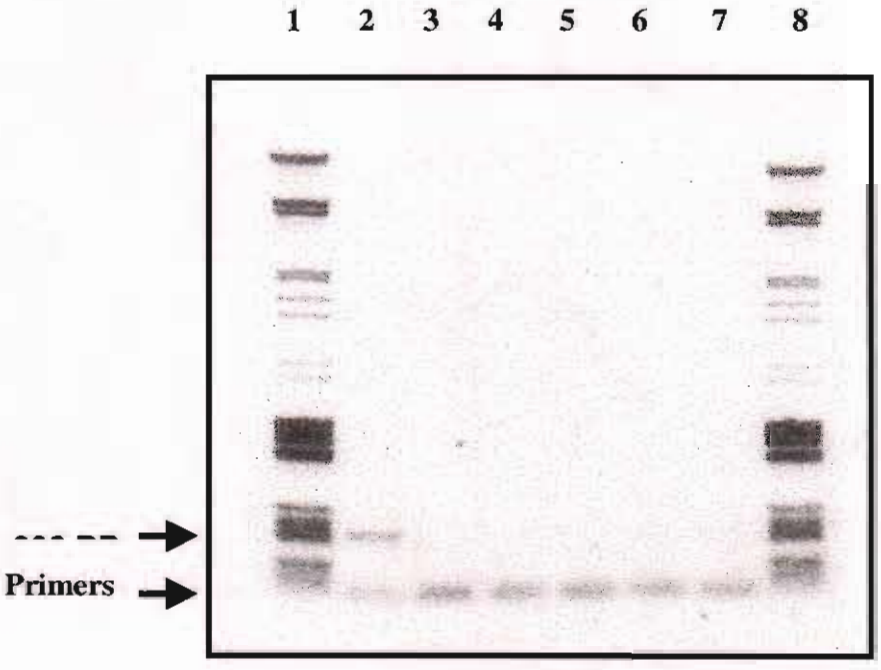


Figure 19: Lane 2 - Amplified 200 bp DNA fragment of the 35S CaMV promoter located on the pVDH 394 plasmid. Lane 1 and 8 - Combination of Molecular Weight Markers III and V (Boehringer Mannheim), Lanes 3 – negative control (no template DNA). Lanes 4 to 7 – PCR performed on soy standard extracts, producing no fragment bands.

It was found that the PCR reaction was unsuccessful when extended to detecting the 35S CaMV promoter controlling glyphosate resistance soy in the soy standards purchased from Fluka Biochemicals. The most likely explanation for this is the low concentrations of GM soy in the soy standards – the highest being 2% GM soy. It is also possible that inhibitors of the PCR co-extracted with the DNA extracts and interfered with the reaction. Insufficient time was available to pursue these problems in greater detail.

Chapter 4: Discussion

4.1 Discussion

EU Regulation 258/97 (Concerning Novel Foods and Novel Food Ingredients) and EU Regulation 1139/98 (Concerning the Compulsory Labelling of Certain Foodstuffs Produced from Genetically Modified Organisms) demand that all foods or food products containing genetically modified foods should be labelled as such. As this legislation becomes law in the EU member countries, it will be necessary for each country to introduce standardised methods to detect genetically modified food and food ingredients. This not only applies to all foods sold within the EU, but also to foods imported from outside the EU –including imports from the US, where genetically modified foods are not seen as different and therefore remain anonymous.

The objective of this work was to develop a reliable and rapid method to detect genetically modified foods. When this work was begun in 1997, methods were available to detect GM tomatoes by extracting DNA from leaves, seeds or calli. No published method was available to detect genetically modified tomatoes in tomato fruit or processed foods, such as tomato paste. Similarly, there were very few methods available to detect genetically modified soy.

This work produced a simple and reliable PCR test to detect genetically modified tomatoes in commercially produced tomato paste. Essentially this involved designing primers to detect the NPT II gene and optimising a PCR reaction profile. A simple

method of reliably extracting DNA from tomato paste was also developed that involved the minimum of extraction steps and sample manipulation. Also, the initial steps in the development of a similar assay for the detection of GM soy have been completed.

4.2 NPT II detection in Genetically Modified Tomato Paste

Polymerase chain reaction was the detection method of choice due to its sensitivity and selectivity (Section 1.7.2). Sensitivity was very important, as good quality template DNA was quite difficult to extract from tomato paste. Difficulties in amplifying genes from tomato paste were reported by Parkes (1999), who found that both the DNA extraction steps and the PCR results for tomato paste were highly variable.

The Neomycin Phosphotransferase II gene was chosen as the gene target to be amplified for several reasons. Firstly, it is the selectable marker inserted into the GM tomato used in the production of the commercially available tomato paste. It is also used as a selectable marker gene in many commercially available genetically modified plant species, so a PCR protocol that works in tomato paste can also be extended to other plant species. Initially, methods for detecting the Neomycin Phosphotransferase II protein were considered. However, it was decided that protein methods were not suitable. Processing involved in the production of tomato paste (*Figure 6*) would cause considerable denaturation of protein constituents of the tomato paste.

DNA is a more robust biomolecule than protein and therefore more likely to survive the rigorous processing steps involved in the production of tomato paste. It has been reported in the literature that the average size of DNA fragments recovered from tomato paste is in the region of 400 bp (Ford *et al.*, 1996). The DNA extracts prepared during the course of this work were of similar fragment length (<1000 bp).

A rapid and simple extraction method was developed based on microwaving the tomato paste sample, followed by diluting the DNA extract to diminish the effects of endogenous PCR inhibitors. This novel approach exposed sufficient template DNA for amplification. A 155 bp fragment of the NPT II gene was successfully amplified from this extract. A restriction digest was performed on pooled samples of the PCR product (Section 2.5). This was carried out to verify that the PCR product produced was in fact the predicted fragment. Two DNA fragments were produced after enzyme digestion, one of 61 bp and one of 94 bp, as expected.

A "dot-blot" (Section 2.7) was undertaken to determine if any DNA could be detected in neat or untreated tomato paste or in DNA extracts. The "dot-blotting" method is qualitative – the result indicates only if the NPT II gene is present or absent. Microwaved GM tomato paste extracts, unextracted tomato paste and DNA extracted using Method A were used as samples. pVDH 394 plasmid DNA was included as a positive control.

All positive controls used showed a significant colour change, however, neither the tomato paste or tomato paste extracts showed any colour change from the background.

This may have been due to the concentration of DNA in the extracts being below the limit of detection of the method.

4.3 CaMV 35S Promoter detection in Genetically Modified Soy Standards

It was decided to design primers to target the CaMV 35S promoter present in the GM soy. This promoter sequence is widely used in gene cassette construction, and therefore a successful test method would have a wide application. The pVDH 394 plasmid uses the CaMV 35S as the promoter and was again used as a positive control in the initial development stages. The CaMV1 and CaMV2 primers successfully amplified a 200 bp DNA fragment.

The objective of isolating DNA from the soy standards was not realised in the course of this work. In this case, attempts to isolate DNA from highly processed soy samples in which the DNA was likely to be significantly degraded were unsuccessful. Methods previously used to detect GM soy have isolated DNA from leaf tissue (Shirai *et al.*, 1998), soybeans (Van Hoef *et al.*,; 1998; Pietsch *et al.*, 1997) or soy products (Wurz et al., 1998). Also, failure of the PCR method could be due to protein contamination in the DNA extract or the absence of sufficiently good quality template DNA.

DNA was extracted from the soy standards using methods outlined in Section 2.2. It was found that extracted DNA was of poor quality when the CTAB method was employed. These observations were in broad agreement with those reported by Zimmermann *et al.*,

(1998). It was also found that the quantities of DNA extracted using the Wizard Genomic DNA extraction kit were lower compared with other methods.

4.4 Limitations to the Methods

The new microwaving method of DNA extraction from genetically modified tomato paste, simplifies the detection of genetically modified foods containing the NPT II gene. The extraction method does not require any solvent use or spin column steps and is reasonably quick. The PCR primers were designed to successfully detect a 155 bp, fragment of the NPT II gene allowing for DNA degradation during processing. The test, from the DNA extraction step to ethidium bromide staining and visualization under ultraviolet light can be completed in less than 3 hours.

Further work will need to be carried out on the method to detect the cauliflower mosaic virus promoter in genetically modified soy. The primers designed during the course of this work were capable of detecting a 200 bp DNA fragment when plasmid DNA was used as a template. However, when this was extended to using soy DNA no amplified product was obtained. This may be due to a PCR inhibitor co-extracting with the DNA, as seen initially with tomato paste. Alternatively, the concentrations of GM soy in the soy standards used are low (varying from 0-2%) and may be beyond the limits of detection of PCR.

The use of PCR as an assay method for detection of specific DNA fragments is well established. However, use of this methodology does require specialised skills. The test methods described above require trained, experienced personnel and specialised equipment. PCR is often subject to interference from food components. It is necessary to develop and validate a *separate* extraction protocol for each foodstuff. This is a formidable task when it is considered that tomato paste may be a component of many pre-prepared foods. Great care is needed in performing PCR analysis as cross contamination from previous assays is always possible. These drawbacks could be a significant barrier to more widespread adoption of this method for routine screening of GM foods. It is likely that screening of GM foods will be carried out in specialised laboratories, at least in the near future.

4.5 Future Applications

The method to detect the NPT II gene in tomato paste as described above, can also be used to detect terminator or promoter sequences, if the appropriate primer pairs are used. A primer directed at the transgene could also be included to make the detection method specific. The inclusion of a normal plant genome target would also act as a control. The use of more sensitised PCR technology (TAQman™, LCR, QB replicase, Biochips and AFM technology) would also increase the detection limits.

In an ideal detection method, several primer pairs could be used – one for the promoter sequence, the terminator sequence, the selectable marker gene (if present) and the trait gene. This will allow a qualitative testing of food samples – an initial test to determine if

the food sample contains GM food or not – followed by a more specific test for the trait gene, which will identify the specific GM plant contained in the food sample. Also, the use of more primer pairs will increase the specificity of the test method, making an accurate determination of the GM plant contained in the sample possible.

If the use of GM foods becomes more widespread, then the monitoring and verification of such foods will be necessary for regulatory and quality assurance purposes. The problem will arise when complex foods containing more than one GM plant material are considered. In that case, detection of several genes in combination will be necessary in order to identify a particular GM component. The potential complexity of such testing is daunting.

A further complication will arise if it becomes necessary to *quantify* levels of GM material in complex foods. The PCR methods considered in this study are qualitative and will only indicate the presence or absence of a gene – not its concentration. For example, legislation in the future may only require food to be labelled as containing GM ingredients if they constitute more than 1% of the total. Using currently available technology, this is difficult to enforce. One such available method - the TAQman™ Sequence Detection System from Perkin Elmer (Heid *et al.,* 1996) – monitors the production of a fluorescent probe attached to DNA produced during thermal cycling. Another possible method would involve comparing the amplification of an internal standard DNA fragment to that of the target gene.

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Appendix 1: Equipment and Solutions

A.1.1 Instruments and Equipment

- Hybaid 'Express' Thermocycler, Hybaid 'Fast track' mini gel
- Spectronic 101 UV spectrophotometer, UVi Tec Transilluminator, Polaroid Gel Cam, Fisons FEC 570 power pack, Fisons FEC 360 gel box
- The Dot-Blot manifold was manufactured by Millipore, France
- Microwave Phillips ' Space cube' 730.

A.1.2 Solutions

All solutions were autoclaved before use and stored at room temperature. The pH was adjusted as necessary.

Buffers used for DNA extraction as described in Section 2.2

Extraction Buffer A (see Section 2.2.1)

100 mM Tris-HCl pH 8

1 M sodium chloride

20 mM EDTA

2% (w/v) SDS

500

µg/ml proteinase k, added after autoclaving.

Cetyltrimethylammonium bromide (CTAB) Buffer pH 8 (see Section 2.2.2)

1% CTAB

50 mM Tris HCl

0.7 M NaCl

10 mM EDTA

0.5% PVP

0.1% 2-mercaptoethanol (added after autoclaving, just before use)

CTAB Extraction Solution (see Section 2.2.3)

2% (w/v) CTAB

100 mM Tris HCl, pH 8.0

20 mM EDTA, pH 8.0

1.4 M NaCl

Stored at Room Temperature after autoclaving.

CTAB/NaCl Solution (see Section 2.2.3)

10% CTAB

0.7 M NaCl

Stored at Room Temperature after autoclaving.

CTAB Precipitation Solution (see Section 2.2.3)

1% (w/v) CTAB

50 mM Tris HCl, pH 8.0

10 mM EDTA, pH 8.0

Stored at Room Temperature after autoclaving.

Buffers used in Dot Blot Assay (Section 2.7)

20X SSC

3 M NaCl

0.3 M trisodium citrate.$2H_2O$

Adjust pH to 7.0 and diluted as appropriate.

Buffers for Immunological Detection of DIG-Labelled DNA

Buffer 1: 10 mM Tris pH7.5

150 mM NaCl

Buffer 2: 10% Blocking Solution in Buffer 1.

Buffer 3: 100 mM Tris pH 9.5

100 mM NaCl

50 mM $MgCl_2$

Buffer 4: 10 mM Tris pH 8.0

1 mM EDTA.

TAE electrophoresis buffer (50X)

242 g Tris base

57.1 ml glacial acetic acid

37.2 g EDTA sodium salt

H_2O to 1 litre.

TE buffer (10X)

10 mM Tris HCl pH 7.5

1 mM Na_2 EDTA

High-Salt TE Buffer

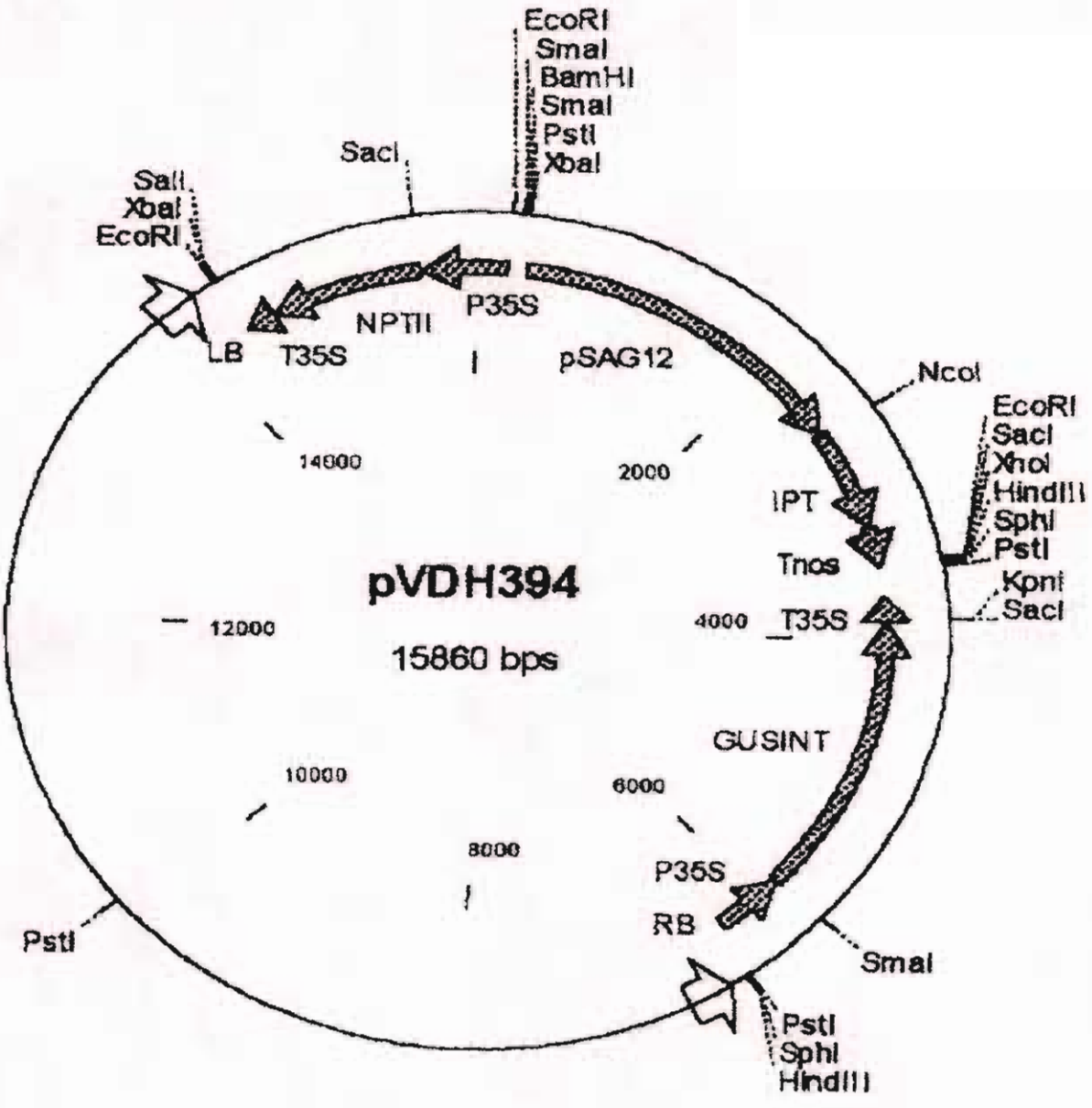
10 mM Tris HCl pH 8.0

0.1 mM Na_2 EDTA

1 M NaCl

Appendix 2: Map of pVDH 394 plasmid

Map of pVDH 394 Plasmid used as a positive control for the detection of both the NPT II and 35S CaMV promoter genes. Kindly supplied by Prof. P. Dix NUI Maynooth.



Appendix 3: Publications

FEATURE

Detecting genetically modified foods

O BOYCE, G BURKE & G HENEHAN

Genetically modified plant foods are increasingly arriving on the market. From the point of view of the consumer or regulator a genetically, modfied plant looks and tastes like one that has not been modified How then can you tell if a plant has been genetically modified? This article reviews some of the test methods that can be used.

Introduction

Man has selectively bred crops for tens of thousands of years. Using classical breeding methods he has increased productivity, disease resistance and improved the taste of his food. In recent years this process has been accelerated by the application of plant biotechnology techniques. In particular, the food industry has been revolutionised by the development and commercialisation of genetically modified (GM) foods. In the United Kingdom (UK) at the moment, there are 15 GM crop products - ranging from fresh FLAVR SAVR™ tomatoes to oil from modified cotton - and 8 food enzymes and cultures approved for sale. There are also 8 GM products currently under consideration for approval (Biotechnology Club Bulletin, 1998). Many more genetically modified foods have been submitted for approval or are in advanced stages of research. The advent of GM foods is, arguably, the most significant development in Food Science in recent years. However, consumers have been alarmed by safety and environmental impact concerns surrounding this new technology (Robinson, 1997). This has led to calls for new legislation requiring the labelling of foods containing GM material.

Genetically modified food looks and tastes exactly like non-modified food. This leaves the customer and the food producer without a way of distinguishing one from the other. As European Union (EU) legislation now obliges all genetically engineered food to be labelled, this presents a problem. Therefore, it is necessary to have *a simple,* reliable, and cost effective method of detecting genetically modified food.

Olivia Boyce, G Burke and Dr Gary Henehan, the Dublin Institute of Technology, Cathal Brugha St, Dublin 1, Ireland, fax: +3531 874 2179.

What is genetically modified food?

In general, genetically modified food refers to a plant that has had a piece of foreign DNA (known as a transgene) inserted into its genome. The plant treats the new piece of DNA as its own and the new DNA confers a beneficial trait on the plant. For example: Round Up Ready soya beans are not killed by the herbicide glyphosate because they have been given a transgene that can inactivate glyphosate. There are two common methods for getting foreign DNA into plant cells.

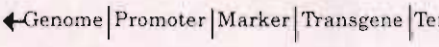
(i) Vector Mediated Transformation; Biological vectors such as *Agrobacterium tumefaciens* are used. This bacterium can invade a plant cell and insert a piece of foreign DNA into its genome.

(ii) Microparticle Bombardment; Microparticles of gold or tungsten are coated with the transgene. The target plant is bombarded with the coated microparticles at 1000 to 2000 ft/sec using gunpowder or helium to provide the blast. The particles can penetrate the cells without killing them, thereby inserting the transgene into the cells (Sanford *et al* 1987)

Promoters, terminators and marker genes.

In addition to the inserted gene that confers the beneficial trait on the plant the transgene construct that enters each plant cell will often contain a marker gene, a promotor sequence and a terminator sequence (Figure 1).

Figure 1: Schematic of a typical transgene construct in a genetically modified plant.



Each of these genetic elements has a specific function;

(i) The promoter regulates the transcription of the transgene. Most approved modified plants use the 35S promoter from the cauliflower mosaic virus (P-CaMV 35S), or its derivatives (Kay *et al* 1987). Another common promoter is the nopaline synthase gene from *Agrobacterium tumefaciens* (P-nos) (An 1986)

(ii) The terminator sequence terminates transcription of the inserted transgene. The most common terminator is the nos3' terminator from the *Agrobacterium tumefaciens* nopaline synthase gene.

(iii) The marker gene is present in many, but not all, GM plants. The most common is the gene that confers resistance to the antibiotic kanamycin. This gene codes for a neomycin phosphotransferase enzyme that inactivates kanamycin. This gene allows plants carrying the gene construct to grow in the presence of kanamycin. The marker gene is present because the technology of introducing genes to plants requires a selection procedure for plants that have acquired the transgene construct.

(iv) The gene carries the beneficial trait e.g. insecticide resistance, pest resistance etc. Often, but not always, this gene will code for an enzyme or protein that confers a benefit on the plant.

Genetic modified plants - the controversy

Genetic modification is new technology, little understood by the public. Concerns have been expressed about the following (Snow and Palma 1997).

- The safety and allergenicity of the foreign proteins in the plant. The new proteins expressed in the plant could cause allergic reactions in certain individuals or might in themselves be toxic.
- The spread of genes across species barriers could alter evolution rates and pose a threat to biodiversity.
- There is concern that genes from genetically engineered plants could spread into closely related species in the field by cross-pollination and produce 'Superweeds'. This is especially relevant in plants engineered to be herbicide resistant.

Legislation

Consumer concerns have culminated in the introduction of EU directives to control the release and labelling of GM foods and food products. Directive 90/219/EEC deals with the contained use of genetically modified microorganisms, while Directive 90/220/EEC deals with the deliberate release of genetically modified organisms into the environment (Pierce, 1997). In the future, as these directives become legislation in the member countries, it will be necessary to have means of detecting GM plants and ingredients entering the food chain. Some of these detection methods are reviewed below.

Detection methods

A genetically modified plant differs from its non-modified counterpart in a few respects. The modified plant contains DNA coding for: the gene conferring the beneficial trait, the marker gene, the promoter and terminator (see Figure 1). As well as the DNA differences, the modified plant may also express an enzyme or protein that confers the beneficial trait and an enzyme that confers antibiotic resistance. A detection method has to exploit these differences.

Many GM plants will have the same promoter gene, the same terminator gene and the same antibiotic resistance marker gene but will differ in the gene conferring the beneficial trait. A detection method that analyses for example, the antibiotic resistance marker gene will detect all modified plants containing this gene. Similarly a detection method for the promoter or terminator sequences will detect all GM plants that have used these genetic elements.

There are two main classes of method that can be used for detecting GM foods, protein-based detection methods and DNA-based detection methods. The target molecules that are detected by each approach are shown in Table 1.

Protein detection

There are problems with the detection of proteins expressed in GM foods. The proteins are often present in small amounts which precludes all but the most sensitive methods of detection. The most significant problem is, however, that proteins are generally denatured by the heating involved in the processing of many foods (Fuchs *et al.*, 1993, Fuchs and Astwood, 1996). Denatured proteins are not amenable to most of the common methods of protein detection.

In the cases where the protein is not denatured the most common methods of detection are:

(i) Direct Activity Assay - Where the protein is an enzyme its activity can be measured directly. This method has been applied to the detection of the neomycin phosphotransferase enzyme expressed in many GM plants (Ramesh and Osborne, 1991, Staebell *et al.*, 1990). However, this method suffers from poor levels of activity and is prone to interference from other enzymes in the plant (see Freigen *et al.*, 1985). This method will not detect denatured protein.

(ii) Immunological Methods - These are based on the reaction between an antibody and its antigen - in this case, the protein to be detected. Widely used and versatile this method is the most sensitive of the protein detection methods (Baszczynski, 1989', Henderson *et al.*, 1991). Usually this method will not detect denatured proteins.

Table 1

Protein Targets	*DNA Targets*
The beneficial protein/enzyme	The gene for the beneficial trait
The enzyme coded by the antibiotic resistance gene	The antibiotic resistance marker gene resistance marker gene
	The promoter sequence
	The terminator sequence

(iii) Electrophoresis Methods - Polyacrylamide gels are used to separate the proteins. The protein of interest is then identified by reacting it with suitably labelled substrates (Freigen *et al.*, 1985). Background protein in the crude tissue extracts often complicates this analysis.

Overall, detection of expressed proteins unique to GM plants is only useful for unprocessed plant foods. For example, neomycin phosphotransferase is detectable in tomatoes but *not* in tomato paste. However, it is important to note that protein detection methods are often more rapid than DNA detection methods and may be the method of choice for unprocessed GM foods.

DNA detection

DNA detection methods have the advantage in that DNA is more robust than protein and can survive many food-processing steps. The success of many DNA methods depends on obtaining a high quality DNA extract from the plant to be tested. Among the methods of detecting specific DNA sequences are;

(i) Probe Hybridisation - The formation of sequence-specific base-paired duplexes by nucleic acid species. DNA probes are immobilised on an inert support and incubated with the sample. Hybridised probes are visualised by autoradiography or chemiluminescent methods (Platt *et al.*, 1987). The method is prone to interference and false positives. To date, this method has been little used for detection of GM Foods.

(ii) Polymerase Chain Reaction (PCR) - This method is by far the most widely used for the identification of GM Foods. PCR can be used to detect one, or several of the genes inserted into the plant, singly or simultaneously (see McGarvey *et al.*, 1991, Xing *et al.*, 1996, Greiner *et al.*, 1997, Padegimas *et al.*, 1993). It is currently the method of choice for GM food identification. The method allows *in-vitro* amplification of DNA sequences from small quantities of target DNA (Hamill *et al.*, 1991). The amplified DNA is then visualised by electrophoresis and compared with molecular weight standards. It is generally recognised as the most sensitive, accurate and versatile method to date.

Despite its popularity the PCR method does have some drawbacks. One of the most important determinants of successful detection of a gene in a food product is the *quality* of the DNA: the DNA preparation must be free of interfering substances and inhibitors of the PCR reaction. Thus, certain salts and proteins found in food products have been reported to be PCR inhibitors (see Ruano *et al.*, 1992, Bickley *et al.*, 1996). It is also necessary that the average base pair length of the DNA extracted from the plant be larger than the length of the target DNA to be detected. Prolonged heating of foodstuffs during processing fragments DNA into small piece. The length of these pieces depends on the degree of processing. PCR will not work on DNA fragments that are shorter than the target sequence.

In addition to the above both false positives and false negative results have been a problem with PCR. False positives arise from carry-over contamination of samples from previous PCR assays. This can be avoided by so called pre-amplification sterilisation (Longo *et al.*, 1990). False negatives arise when the amount of DNA to be detected is a small percentage of the total DNA and sensitivity is an issue. This can be avoided by including a positive control targeted to a sequence known to be present in the test material at a similar concentration to the GM gene.

While all the problems above can be circumvented, perhaps the most significant barrier to more widespread adoption of PCR testing methods is the lack of familiarity with this new technology. This is especially true in industrial laboratories where trained personnel may not be available.

Commercial testing of GM plants

At the moment it is possible to send samples of GM food to be tested to a number of laboratories. All of these testing facilities use the PCR reaction as a basis for their analysis although their methods may detect different genetic elements. Most use more than one set of primers to identify different genes to improve their reliability. GM analysis services are available from the following: Central Science Laboratory, Norwich, UK; Leatherhead Food Research Association, UK; Kantonales Labor BaselStadt, Switzerland; Genetic ID, Iowa, USA; Hanse Analytik GmbH, Germany. These services between them offer analysis for GM corn chicory, potato, rapeseed, tomato, squash, papaya, cotton, tobacco and sugar beet.

In addition to these services there are assay kits commercially available for detection of a variety of GM plants. Kits contain the primers, control DNA, reagents and buffers necessary for PCR analysis of specific target sequences. Kits are available from Hanse Analytik GmbH, Germany and Verigene, Dublin, Ireland.

Conclusion

The detection of genetically modified food is going to become increasingly important to the food industry and regulatory bodies as the number of approved products increases. Due to consumer concern and forthcoming EU legislation, it will be necessary in the future to have recognised and validated methods to distinguish genetically modified food from non-modified food. The cost of food control is likely to be significant unless inexpensive and widely applicable methods of GM food identification are developed. Such methods are likely to be based around the PCR technique.

At the moment, services are available commercially, which will detect GM food, but it may