DETECTION OF GENETICALLY MODIFIED POTATOES BY THE POLYMERASE CHAIN REACTION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ABUBAKER MUWONGE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

JANUARY 2005

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Canan ÖZGEN
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Dilek SANİN
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Dr. Remziye YILMAZ
Co-Supervisor

Assoc. Prof. Candan GüRAKAN
Supervisor

Examining Committee Members

Prof. Dr. Mahinur AKKAYA	(METU, Chem)	
Assoc. Prof. Dr. Candan GÜRAKAN	(METU, Fde)	
Prof. Dr. Sabahattin ÖZCAN	(Ankara Univ. Ziraat Fak)	
Prof. Dr. Zeki KAYA	(METU, Bio)	
Dr Remziye Yilmaz	(Agric. Ministry)	

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Abubaker Muwonge

ABSTRACT.

DETECTION OF GENETICALLY MODIFIED POTATOES BY THE POLYMERASE CHAIN REACTION (PCR)

Abubaker Muwonge

M.Sc., Department of Biotechnology

Supervisor: Assoc. Prof. Dr. G. Candan Gürakan

Co-supervisor: Dr. Remziye Yılmaz

January 2005, 95 pages

Quite a number of important crops have been genetically modified with genes for agronomically important traits, such as insect and viral resistance.

As the numbers of genetically modified foods continue to increase on the market, the need for rapid development of GMO detection methods is indispensable.

This study was carried out to detect if genetically modified potatoes exist on food market in Turkey. Thirty samples from different places were collected. Using a DNA based PCR method, potato samples were examined for the presence of 35S promoter, Nos terminator, neomycin phosphotransferase (nptII) genes, and synthetic cry3A gene which is the general transgene in all approved Newleaf transgenic potato lines.

The experimental design of this study was to detect Newleaf insect resistant lines. In 11 samples at least one genetic element was detected. Sample R from Ankara has shown to be belonging to Newleaf insect resistant lines. Since 35S promoter was not detected in samples M3, 14 and F1, it is proposed that they are belonging to Newleaf

virus and insect resistant lines (Newleaf plus or Newleaf Y). Although Nos terminator was not detected in samples H2, Z2 and D, cry3A fragments amplified in those samples have been verified that they are from the synthetic cry3A regions of Newleaf lines.

The detected synthetic cry3A gene in GM potatoes was amplified by specific primers, which cannot amplify *Bacillus thuringiensis tenebrionis* natural cry3A gene. In addition, the authenticity of the synthetic cry3A PCR products were confirmed by both sequencing and restriction digestions.

Our results showed that genetically modified Newleaf potatoes exist in food market in Turkey. Further studies by accredited laboratories are strongly recommended.

Key words: genetic modification, potato, PCR, detection, synthetic cry3A.

ÖZ

GENETİK OLARAK DEĞİŞTİRİLMİŞ PATATESLERİN POLİMERAZ ZİNCİR REAKSİYONU İLE TANISI

Abubaker Muwonge
Yüksek Lisans Biyoteknoloji Bölümü
Tez yöneticisi: Doç. Dr. G. Candan Gürakan
Yardımcı tez yöneticisi: Dr. Remziye Yılmaz

Ocak 2005, 95 sayfa

Pekcok sayıda önemli ürün böcek ve virüs direnci gibi tarımsal açıdan önemli genlerle genetik olarak modifiye edilmiştir.

Genetik olarak değiştirilmiş gıdaların pazardaki sayısı artmaya devam ettikçe, hızlı GMO tanı yöntemlerinin geliştirilmesine duyulan ihtiyaç göz ardı edilemez.

Bu çalışma Türkiye de gıda pazarında gentik olarak değiştirilmiş patates olup olmadığını araştırmak amacı ile gerçekleştirilmiştir. Farklı yerlerden 30 numume toplanmıstır. DNA bazlı metot kullanılarak patates örnekleri 35S promotor, nos terminatör, neomisin fosfotransferaz (nptII) geni bölgelerinin ve tüm onaylı Newleaf transgenik patates hatlarında bulunan genel transgenik gen olan sentetik cry3A genin varlığı incelenmiştir.

Bu çalışmanın deneysel tasarımı Newleaf böcek direnci hatlarının bulunması amacını taşımaktadır. 11 örnekte en azından bir genetik element bulunmuştur. Ankaradan alınan örnek R`ın Newleaf böcek direnci taşıdığı tespit edilmiştir. 35S promotor bölgesinin M3, 14 ve F1 örneklerinde bulunamaması nedeniyle, bu örneklerin Newleaf virüs ve böcek direnç (Newleaf Plus veya Newleaf Y) hatlarına ait olabileceği ileri sürülmüştür.

H2, Z2 ve D örneklerinde Nos terminatör bölgesi bulunamamasına rağmen, bu örneklerde çoğaltılan cry3A fragmantının Newleaf hatlarında bulunan sentetik cry3A bölgesine ait olduğu kanıtalanmıştır.

Genetik modifiye patateslerde Basillus thuringiensis tenebrionis (Btt) doğal genini çoğaltamayan spesifik primerlerle sentetik cry3A geni tanısı yapılmıstır. Buna ek olarak sentetik cry3A` nın polimeraz Zincir reaksiyonu (PZR) ürünlerinin özel yapısı hem DNA sekanslama ve kesici enzimler yoluyla doğrulanmıstır.

Sonuçlarımız genetik modifiye Newleaf patateslerin Türkiye de yiyecek marketlerinde bulunduğunu göstermektedir. Akredite laboratuvarlar tarafından yürütülecek ileri çalışmalar önerilmektedir.

Anahtar kelimeler: genetik modifikasyon, patates, polimeraz zincir reaksiyonu, sentetik cry3A

ACKNOWLEDGEMENT

I wish to express my sincere and heartfelt gratitude to my supervisor Assoc. Prof. Dr. Candan Gürukan for her guidance and support to bring this work to completion. She has always been very supportive right from the beginning of my thesis work. Besides, she at all times attended to listen to me; even at times she was too busy with other works. Thank you very much; I wish I would have the opportunity to work with such a dedicated and considerable personality in the future.

I also wish to thank my co-supervisor Dr. Remziye Yilmaz for her advice and contribution.

Thanks to Prof. Dr. David Douches and Ms Susannah Cooper of Michigan State University (Crop and Soil science Department) for providing the positive control for this study. I also thank Dr. Jaccuad Etienne, Research scientist, Nestle research center Switzerland for providing more positive controls. My sincere gratitude is owed to Prof. Dr. Mahinur Akkaya for providing restriction enzymes used in this study.

Am very grateful to my laboratory mates, Gamze Aydin, Zeynep Sönmezalp, among others in laboratory AZ-07 for their continued support right from the time I entered the laboratory. Minus them; I guess my work would have taken longer. My thanks also go to Gökhan Duruksu, Aysun Cebeci Aydın and Adnan Al-Halsi for their support during my study period.

I am very grateful to my parents and relatives for their love and care, they rendered to my family right from the time I left home. To my parents; Mr. Edirisa Nadibanga Muwonge, Mrs. Nakibule Marium and Mrs. Namutebi Margaret, am greatly indebted for your care and support.

My special thanks and gratitude go to my loving wife Ms Nakiwala Aisha Sembatya Muwonge for accepting to leave her and go to attend school. Ever since I left home, it has been very difficult and challenging but you have been very supportive, caring, compassionate, and trustful at the very trying moments. Please I say thank you very much. You believed in me and encouraged me to work hard and be back home as soon as possible.

I also wish to thank my housemates Mr. Kadangu Habib and Mr. Kinje Menya Bakari for all the support, good brotherhood, and respect you accorded me.

Finally I wish to thank Islamic Development Bank for their sponsorship and I pray that this support continues to other deserving students.

This work is dedicated to my Dad, Mr. Edirisa Nadibanga and my loving wife Mrs. Aisha Nakiwala Muwonge.

TABLE OF CONTENTS

PLAGIARISM……………………………………………………………………….…iii

ABSTRACT……………………………………………………………………......iv

ÖZ……………………………………………………………………………….…vi

ACKNOWLEGMENT ……………………………………………………...…...viii

DEDICATION…………………………………………………………………..x

TABLE OF CONTENTS……………………………………………………….xi

LIST OF TABLES……………………………………………………………..xv

LIST OF FIGURES……………………………………………………………xiv

ABBREVIATIONS…………………………………………………………....xvi

CHAPTERS

1. INTRODUCTION

1.1 Genetically modified organism……………………………………..……1

1.1.1. Global production of transgenic crops………………………………...3

1.1.2. The art of genetic engineering……………………..…………………..5

1.1.3. GM potatoes on market…………………………………………….….6

1.1.4. Benefits of GM crops……………………….………...………10

1.1.4.1. Increased food production……………….…………….…11

1.1.4.2. Improved food quality……………………………….…...12

1.1.4.3. Improved nutritional quality……………..…………………12

1.1.4.4. Improved protein quality……………………………………13

1.1.4.5. Improved quality and quantity of animal products….………14

1.1.4.6. Health benefits………………………………………………14

1.1.4.7. Environmental benefits……………………………………...15

1.1.5. Potential concerns of GM crops………….…………………………...16

1.1.5.1. Antibiotics resistance…………….……………………….16

1.1.5.2. Potential toxicity……………………….………………16
1.1.5.3. Environmental concerns………………….………………16
1.1.5.4. Potential allergies…………………………………………...17
1.1.5.5. Patenting of GM crops…………………….…………..…..17
1.1.5.6. Threat to crop genetic diversity…………………………..…18
1.1.5.7. Religious, cultural and ethical concerns……………….…18
1.1.5.8. Concerns of animal groups and organic farmers……………18
1.1.5.9. Fear of unknown…………………………..…………...19
1.2. Detection of GMO….……………………………………….……19
1.2.1. DNA based methods……………………..…………………21
1.2.2. Protein based methods……………………….…………………..24
1.3. Regulation of foods derived from GMOs……………………………….26
1.3.1. Regulations in EU…………………….………………………….26
1.3.2. Regulation in USA…………………………..……………..27
1.3.3. Regulation in Canada…………………………………………...28
1.3.4. Regulation in Japan……………………………………………..28
1.3.5. Regulation in Turkey…………………………….………28
1.3.6. Regulation in Australia…………………..…………………29
1.3.7. Regulation in South Africa……………………………………29
1.4. Safety assessment of foods derived from GM crops……………………31
1.4.1. Substantial equivalence…………………………………….31
1.5. Impact of GM crops on the third world……………….………………..32
1.6. Consumers awareness and perception of GMOs………..………………34
1.7. Objectives of the study………………………………………….………35

2. MATERIALS AND METHODS

2.1. Potato materials…………………………………………….……...36
2.2. Reference materials…………………………………...………………36
2.3. Chemicals, buffers and solutions……………….…………………….37
2.4. DNA extraction……………………………….....………………..37
2.4.1. Potato DNA extraction……………………………………..37
2.4.2. Btt chromosomal DNA isolation……………………………...39

2.5. Determining the DNA purity……………………………………………39
2.6. Agarose gel electrophoresis……………………………..……………40
2.7. Primer design…………………………………………………………41
2.8. Polymerase chain reaction (PCR)…………………………….………42
2.8.1. Patatin specific PCR………………………………….………43
2.8.2. GMO screening PCR systems……………………..………44
2.8.2.1. Screening for P-35S ..…………………………….…...44
2.8.2.2. NptII specific PCR………………….…………........45
2.8.2.3. Nos terminator specific PCR…………………….…46
2.8.3. Synthetic cry3A specific PCR……………………….…….47
2.8.4. Amplification of Btt DNA……………………………………48
3. RESULTS AND DISCUSSIONS
3.1. DNA extraction…………………………………………………………50
3.2. Patatin specific PCR…………………………………………..……...51
3.3. NptII specific PCR……………………………………….…..…….53
3.4. P-35S specific PCR…………………………………………..………55
3.5. Nos3-T specific PCR…………………….……………………...56
3.6. Synthetic cry3A specific PCR……………………………………..58
3.7. Specificity of PCR systems……………………………………..61
3.8. Confirmation of PCR products………………………………….……62
3.9. Sensitivity of synthetic cry3A PCR system…………….……………68
3.10. Interpretation of results………………………………………………...69
4. CONCLUSIONS…………………………………………………………74
REFERENCES…………………………………………………………..76
APPENDICES……………………………………………………………91
Appendix A. CHEMICALS AND SUPPLIERS…………...................................91
Appendix B. BUFFERS AND SOLUTIONS……………………………….92
Appendix C. PRIMER BINDING SITES…………….…………………………94

LIST OF TABLES

Table 1.1. Sequence details of DNA constructs……………………………………8
Table 1.2. Genetic elements transformed in Newleaf events…………………………9
Table 1.3. DNA and protein based methods compared……………………………..25
Table 1.3. Approvals status of GM potatoes…………..…………………………….30
Table 2.1. Potato samples used in the study…………………………………………37
Table 2.2. List of primers used in the study…………………………………..……..42
Table 2.3. Concentrations of PCR components for patatin gene…………….……...43
Table 2.4. PCR conditions for patatin gene……………………………………….…43
Table 2.5. Concentrations of PCR components for 35S promoter…………………..44
Table 2.6. PCR conditions for 35S promoter……………………………………….45
Table 2.7. Concentrations of PCR components for nptII gene……….………………45
Table 2.8. PCR conditions for amplification of nptII gene…………………………46
Table 2.9. Concentrations of PCR components for Nos terminator..……….………46
Table 2.10. PCR conditions for amplification of Nos terminator………..…………47
Table 2.11. Concentrations of PCR components for synthetic cry3A………………47
Table 2.12. PCR conditions for amplification of synthetic cry3A gene……………48
Table 2.13. Concentrations of PCR components for Btt DNA……………………...48
Table 3.1. Lanes for genomic DNA agarose gel…………………………….…………51
Table 3.2. Lanes for patatin specific PCR products…………………….………...52
Table 3.3. Lanes for nptII PCR products…………………..……………………...54
Table 3.4. Lanes for 35S promoter PCR products………………………….……..56
Table 3.5. Lanes for Nos terminator PCR products……………….……………...57
Table 3.6. Lanes for synthetic cry3A specific PCR products………………………..59
Table 3.7. Comparison of sequences of fragments generated with synthetic cry3A specific primers with natural cry3A sequences…………………………………..…63
Table 3.8. Summary of all PCR results………………………………………...70

LIST OF FIGURES

Figure 1.1. Global area of transgenic crops……………………………………….…..4
Figure 1.2. Transgene design construct……………………………………………6
Figure 1.3. DNA construct map used for transformation of Newleaf Atlantic lines…...7
Figure 1.4. DNA construct map used for transformation of Newleaf Y lines………...7
Figure 1.5. DNA construct map used for transformation of Newleaf Plus lines……...8
Figure 3.1. Genomic DNA agarose gel…………………………………………...50
Figure 3.2. PCR results for patatin gene…………………………………………..52
Figure 3.3. Specificity of patatin primer set Pat-F/Pat-R……………………………53
Figure 3.4. PCR results for nptII gene…………………………………..………...54
Figure 3.5. PCR results for 35S promoter……………………………..…………..55
Figure 3.6. PCR results for Nos3 terminator ……………………………..…………57
Figure 3.7. PCR results for synthetic cry3A gene………………………………..58
Figure 3.8. Specificity of PCR assays for transgenic vs non-transgenic potatoes…….61
Figure 3.9. Sequence alignment of sample H2 fragment with synthetic and natural cry3A sequences……. ..……………………………………………………………..63
Figure 3.10. Sequence alignment of sample R fragment with synthetic and natural cry3A sequences………………………………………………………………..65
Figure 3.11. Sequence alignment of synthetic cry3A and naturally occurring cry3A gene showing PvuII restriction site……………………………………..66
Figure 3.12. PvuII digestion of synthetic cry3A amplicons…………………………67
Figure 3.13. Specificity of primers 3A-F and 3A-R for synthetic cry3A……………68
Figure 3.14. Absolute limit of detection in PCR of synthetic cry3A……………..……69

ABBREVIATIONS

GM	: Genetic modification
GMO	: Genetically modified organism
GMOs	: Genetically modified organisms
GE	: Genetic engineering
DNA	: Deoxyribo nucleic acid
cDNA	: Complementary deoxyribo nucleic acid
CPB	: Colorado potato beetle
PLRV	: Potato leafroll virus
PLRV rep	: potato leafroll virus replicase
ORF1	: Open reading frame 1
OFR2	: Open reading frame 2
PVY	: Potato virus Y
PVY-O	: Potato virus Y strain O
PCR	: Polymerase chain reaction
Cp	: Coat protein.
Bt	: Bacillus thuringiensis
Btt	: Bacillus thuringiensis tenebrionis
USA	: United States of America
USDA	: United States department of agriculture
EPA	: Environmental protection agency
Fig	: Figure
FDA	: Food and drug Authority
OECD	: Organization for economic cooperation and development.
WHO	: World Health Organization
FAO	: Food and Agriculture Organization
APHIS	: Animal and plant inspection service
MAFF	: Ministry of Agriculture, Food and Fisheries
MHLW	: Ministry of Health, Labor and Welfare

FSANZ	: Food Standards Australia New Zealand
NOS-T	: Nopaline synthase terminator
35S-P	: 35S promoter
P-Ssu	: Promoter, small subunit
RB	: Right border
LB	: Left border
NptII	: neomycin phosphotransferase II
P-FMV	: Promoter Figwort Mosaic Virus
hsp	: heat shock protein
GBSS	: Granule bound starch synthase
rPST	: recombinant porcine somatotrophin
rBST	: recombinant bovine somatotrophin
HIV	: Human immune deficiency virus
BSE	: Bovine spongiform encephalopathy
EC	: European Commission
EU	: European Union
Taq	: Thermus aquarticus
ELISA	: Enzyme linked immunosorbent Assay

CHAPTER 1

INTRODUCTION

1.1. Genetically Modified Organism (GMO)

Recent advances in cell and molecular biology has opened new avenues for the production of genetically modified organisms. Genetic modification can be defined as a process where the genetic material (DNA) is altered in a way that does not occur naturally by mating or natural recombination. It's accomplished by recombinant DNA techniques. An organism whose genetic material has been altered using such means is said to be genetically modified, genetically engineered or transgenic. The inserted DNA is translated and new protein expressed, giving an organism a new characteristic/trait.

A series of genes responsible for agronomically important traits such as insect and viral resistance; stress tolerance; herbicide tolerance, among others have been transferred into several major crops (Taylor et al., 1997).
Maize, tomatoes, soybeans, potatoes cotton, rice, canola, papaya and wheat, are among the genetically modified crops on the market today (Lin et al., 2000). Also various pharmaceutical compounds such as enzymes, monoclonal antibodies, nutrients, hormones, and drugs and vaccines are now manufactured in bulk using transgenic organisms.

The development of GM crops is proceeding rapidly and several transgenic crops are already in large-scale production in some countries (ISAAA, 2003). GM crops are a promise to increased food production, better pest management, improved food quality, production of pharmaceutical products among others.

United States, Canada, Argentina and China continue to be the leading growers of GM crops, although other countries are starting to follow suit. The total acreage of GM crops has increased since 1995 in both industrial and developing countries (Clive 2003).

Potato (S*olanum tuberosum*) is the 4th most important crop in the world after wheat, corn and rice, in terms of acreage grown, yield and value of the crop (Alexander. et al, 2003). Potato nutrient values include vitamin C, and are particularly useful as source of energy and protein. Among other forms, potatoes can be consumed as potato chips, French fries, or be processed into potato flour, alcohol and potato starch. Annual production approaches 300 million tons with China producing 16% of the total world production (ICP, 2000).

Pathogen control however remains a problem for farmers in many parts of the world. The Colorado Potato Beetle (CPB), Potato LeafRoll Virus (PLRV) and Potato Virus Y (PVY) are the most devastating pathogens causing yield loss of up to 90% in infected plants (Palucha et.al, 1998).

Modification of potato using genetic engineering holds enormous potential to alleviate these problems. The vast majority of transgenic research has centered on creating novel resistance to potato pathogens (Alexander. et al., 2003). Some potato lines have been transformed with insecticidal Cry 3A gene derived from *Bacillus thuringiensis tenebrionis* and viral coat protein to make them resistant to pests and viral infection respectively (BATS, 2003).
Monsanto in 1996, released their potato cultivars branded, ´ Newleaf that were engineered against CPB, PVY and PLRV resistance (Reed et al., 2001).

Despite the benefits GMOs or GM foods come with, some consumers are still very skeptical to accept them, and they demand that GMO should be labeled. GMO labeling requirement has created demand for developing GMO detection methods (Anklam et al., 2002). The basis of every GM detection technology is to find the

difference between an unmodified and transgenic plant. This can be done by detecting the new DNA that has been inserted (DNA based) or the new protein expressed (protein based) or if the protein acts as an enzyme, by using chemical analysis to detect the product of the enzymatic reaction (Gadani et al., 2000).

Polymerase Chain Reaction (PCR), a DNA based method is the most widely used method because it's very specific and sensitive, and unlike proteins, DNA is relatively thermal stable (Anklam et al., 2002). PCR is used to detect gene construct that was inserted into the crop, which could be promoter, terminator, structural gene and/or marker gene.

1.1.1. Global Production of Transgenic Crops

During the eight-year period 1996 to 2003, global area of transgenic crops increased 40 fold, from 1.7 million hectares in 1996 to 67.7 million hectares in 2003, with an increasing proportion grown by developing countries (ISAAA, 2003). The 30% of the global transgenic crop area in 2003 was grown in developing countries where growth continued to be strong (Clive, 2003).

The absolute growth in GM crop area between 2002 and 2003 was almost the same in developing countries (4.4 million hectares) and industrial countries (4.6 million hectares), with the percentage growth as high as 28% in the developing countries compared to 11% in the industrial countries (ISAAA, 2003).
According to ISAAA, 67.7 million hectares of GM crops in 2003 was grown by 7 million farmers in 18 countries, an increase from 6 million farmers in 16 countries in 2002. The increase in area between 2002 and 2003 of 15% is equivalent to 9 million hectares (Fig 1.1).

GM crop area by country; the USA grew 42.8 million hectares (63% of global total), Argentina 13.9 million hectares (21%), Canada 4.4 million hectares (6%), Brazil 3

million hectares (4%), China 2.8 million hectares (4%) and South Africa 0.4 million hectares (1%) (ISAAA, 2003).

The crop area growth rate between 2002 and 2003 was 33% for China and South Africa, Canada with 26%, USA 10% and Argentina 3%. There was strong growth in both Bt and herbicide tolerant maize, and continued growth in herbicide tolerant soybean (ISAAA, 2003).

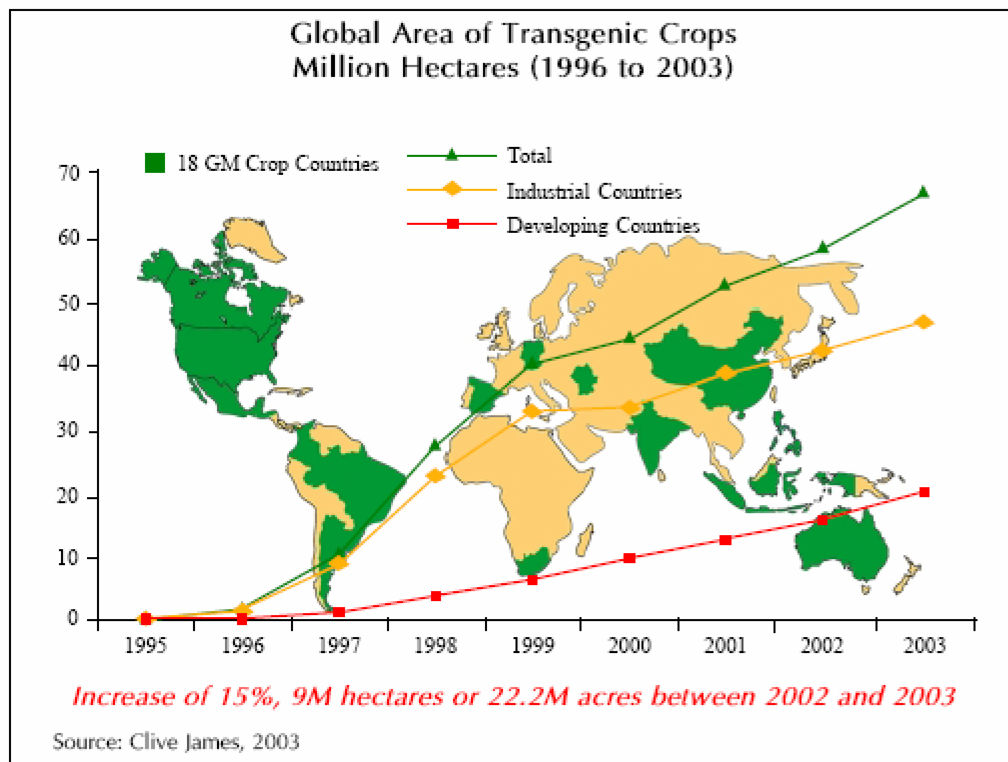


Fig 1.1. Global area of Transgenic crops.

Two countries, Brazil and the Philippines approved planting of GM crops for the first time in 2003. The number of countries growing GM crops increased steadily from 6 in 1996, to 9 in 1998, to 13 in 2001, and 18 in 2003 (Clive, 2003).

ISAAA reported that, globally in 2003, GM soybean occupied 61%, GM maize 23%, transgenic cotton 11% and GM canola 5% of global GM area.

GM crops by trait, herbicide tolerance has consistently been the dominant trait followed by insect resistance. In 2003, herbicide tolerance occupied 73% and Bt crops 18% of the global GM area. The two dominant GM crop/trait combinations in 2003 were: herbicide tolerant soybean occupying 61% of the global total and grown in seven countries; and Bt maize, occupying 13% of global transgenic area and grown in nine countries (Clive, 2003). In 2003, 55% of the soybean, 21% of cotton and 11% of maize planted globally were transgenic (ISAAA, 2003).

1.1.2. The Art of Genetic Engineering

The genetic "code" is the information that determines the nature of the organism stored in DNA. Genes are particular sections of DNA, spaced out along it, which determine the characteristics and functions of the organism (Primrose et al., 2001).

Genetic engineering is a process where genetic material is transferred from one organism to another using recombinant DNA techniques. Genetic material is extracted from a living organism, isolated and manipulated, and either replaced in the same organism, or put into a different one (Primrose et al., 2001).

Genetic engineering involves the following steps;

Step 1. Identification of Trait & Its Source. The entire DNA genome from the source organism is extracted. This DNA sample contains the gene for the desired trait plus the rest of the organism's DNA.

Step 2. Gene Isolation. With the use of gene sequencing and gene mapping, the gene responsible for desired trait is identified, located and using restriction enzymes, the desired DNA fragment is separated from the others.

Step 3. Gene Design. Once the desired gene is isolated, it's designed so that it can be expressed by a different organism. It's put under the control of promoter and terminator so that it will to be correctly expressed (i.e. translated into a protein). A

selectable marker gene is also added to the transgene in order to identify plant cells or tissues that have successfully incorporated the transgene.

Selectable marker genes code for proteins that provide resistance to substances that are normally toxic to plants, such as antibiotics or herbicides.

Fig. 1.2. Transgene design construct.

Step 4. Transformation. It involves inserting transgene into recipient plant genome. Normally undifferentiated cell mass, i.e. callus is used and method of transformation can be biological or chemical/physical. *Agrobacterium* method is most widely used method in plants.

Step 5. Selection. It is aimed at finding if transgene was successfully incorporated and expressed. Selection is done depending on selectable marker that was used during gene design. Only transgenic plants expressing the selectable marker gene will survive when exposed to selection medium.

Step 6. Backcross Breeding: Crossing GE plant with elite plants, then carrying out field trails to verify whether the transgene has been stably incorporated without negative effects to other plant functions, product quality or the intended agro ecosystem. Other Steps relate to Product Safety assessment and Regulations.

1.1.3. GM Potatoes on Market

To combat potato pathogens and diseases, genetic engineering strategies have been sought. GM potato cultivars resistant to CPB, PVY and PLRV were developed by Monsanto and approved for field production, feed and food since 1996 in Australia, Canada, USA, Philippines, Romania and Japan (BATS, 2003). Three GM potato

cultivars were on market by the year 2003; these are Newleaf, Newleaf Plus and Newleaf Y (BATS, 2003).

Newleaf (brand name) was genetically engineered to express the insecticidal protein Cry 3A delta endotoxin derived from *Bacillus thuringiensis tenebrionis* that is highly selective in controlling CPB.

Below is figure showing the DNA construct used to create Newleaf Atlantic lines: ATBT04-6, ATBT04-27, ATBT04-30, ATBT04-31, and ATBT04-36.

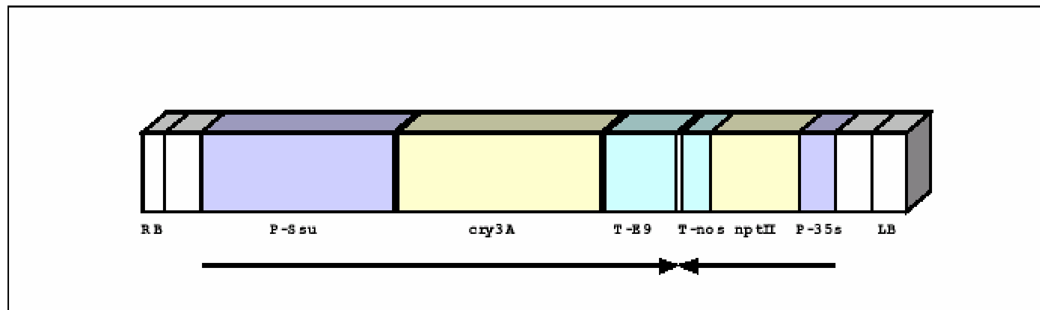


Fig.1.3. DNA construct used for transformation of Newleaf lines, Atlantic.
Sequences details are shown in Table 1.1.

Newleaf Y lines; RBMT15-101, SEMT15-02, SEMT15-15 and HLMT15-46 were engineered for resistance to both CPB and to infection by PVY-O (BATS, 2003). Besides Cry3A delta endotoxin, Newleaf Y lines were also transformed with PVY coat protein.

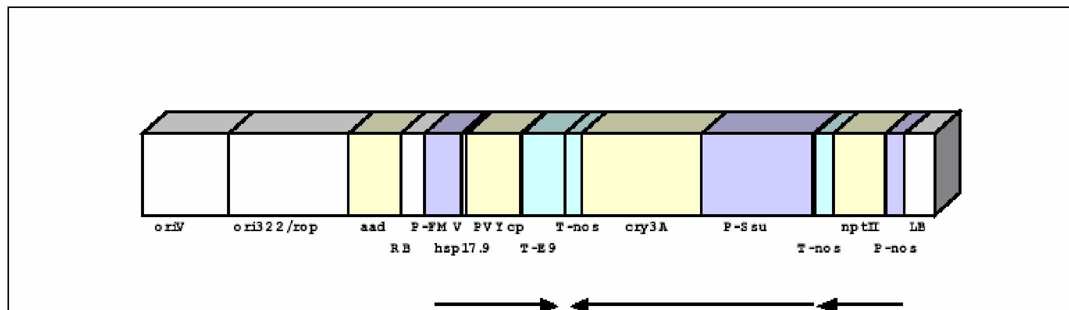


Fig. 1.4. DNA construct map used for transformation of Newleaf Y lines. (Source: BATS, 2003).

Sequence details of the construct are shown in Table 1.1.

Newleaf Plus lines; RBMT21-129, RBMT21-152, RBMT21-350 were genetically engineered for resistance to CPB and PLRV. These lines were transformed with Cry 3A delta endotoxin gene and PLRV replicase gene (PLRV ORF1 and ORF2) (BATS, 2003).

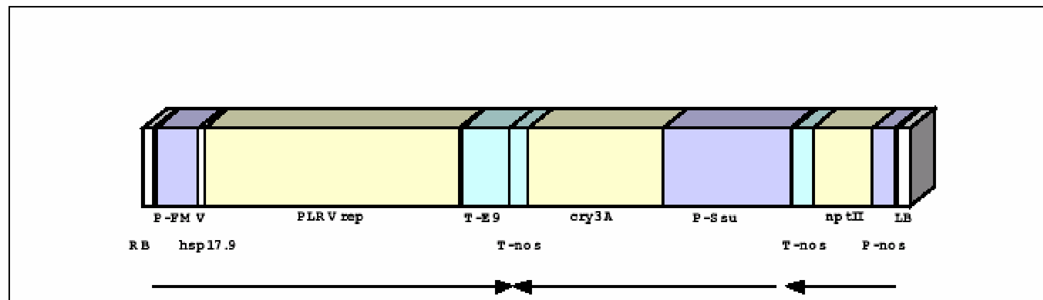


Fig. 1.5. DNA construct map used for transformation of Newleaf Plus lines. (Source, BATS, 2003)

Table 1.1 Sequence details of DNA constructs

Abbreviation	Element-Name	Size (Kb)
RB	Right Border	
P-FMV	Figwort mosaic Virus Promoter	0,57
hsp17.9	Heat shock protein 17.9KD leader sequence	0,077
aad	3 (9)-O- aminoglycoside adenyltransferase	0,79
T-E9	Terminator from Pea	0,63
T-Nos	Nos Terminator	0,26
P-Ssu	Promoter from Arabidopsis thaliana Ssu gene	1,7
nptII	Neomycin phosphotransferase	0,79
P-nos	Nos promoter	0,3
	Cry3A delta endotoxin	1,8
PLRV rep	Potato leafroll virus replicase	3,4
PVY cp	Potato virus Y coat protein	0,81
P-35S	35S promoter	0,32
LB	Left border	

Source: BATS, 2003

The summary of most commonly used genetic elements transformed in ‘’Newleaf`` events is shown in Table 1.2.

Table 1.2. Genetic elements transformed in Newleaf events

	P-35S	T-Nos	NptII	Cry3A	PVY	PLRV
Newleaf events	+	+	+	+	-	-
Newleaf Y events	-	+	+	+	+	-
Newleaf Plus events	-	+	+	+	-	+

+, used; -, not used.

Also some Universities have been at fore front of developing GM potatoes. The Michigan State University together with The Agricultural Biotechnology Support Project (ABSP) released two potato lines for commercial cultivation. These Potato lines Spunta-G2 and Spunta-G3 each was transformed with vector (pSPUD5), including a gene cassette consisting of CaMV35S promoter - Cry5-Bt gene - NOS terminator (Mohammed et al., 2000). The bt-cry5 gene is for resistance against potato tuber moth (*Phthorimaea operculella zeller*). These potatoes are cultivated in Egypt (http://www.msu.edu/douchesd/commercialRelease, 11/12/2004).

Alexander et al (2003) transformed 4*x*-2*x* potato hybrids commonly grown in developing countries with *Bacillus thuringiensis cry3Aa* endotoxin protein gene and the PVY coat protein gene using *Agrobacterium tumefaciens.* Results suggest that transgenes, such as *cry3Aa*, could be expressed in 4*x*-2*x* hybrids to lower costs of production with no significant effect on plant phenotype.

High levels of Resistance to PVY were achieved by transformation potato with coat protein gene (Hefferon et al., 1997) as well as the virally encoded P1 proteinase sequence of PVY^O strain (Maki-Valkama et al 2000; Pehu et al., 1995).

In addition, heterologous immunity to PVY^{O} was achieved through transformation of potato with lettuce Mosaic Potyvirus (LMV) coat protein gene (Hassairi et al., 1998). Using coat protein transformation strategy, researchers have developed transgenic plants resistant to potato Mop-Top virus (Barker et al., 1999) and potato virus X (Spillane et al., 1998). Viral resistance in transgenic plants is based on gene silencing mechanisms (Alexander et al., 2003).

Li et al (1999) combined cry 5 gene with a PVY^{O} coat protein gene to produce lines of cultivar Spunta with high resistance to both tuber moth and PVY infection. Control of late blight (*Phytophthors infestans*), a potato fungal disease has been achieved by engineering fungal glucose oxidase gene from *Aspergillus niger* into potato (Zhen et al., 2000). Developing resistance to the bacterial pathogens causing soft rot and blackleg disease during storage was attempted by engineering antimicrobial genes cecropin B and SB- 37 from *hyalophora cecropia* into potato (Arce et al., 1999).

A GM potato designated line EH92-527-1 with high amylopectin starch was developed for starch production. In this Potato line the genetic modification involved antisense inhibition of the gene encoding granule bound starch synthase protein (gbss), which is responsible for amylose biosynthesis. The starch produced has little or no amylose and consists of branched amylopectin, which modifies the physical properties of the starch. The Scientific Committee on Plants of European Commission carried out safety approval for placing on market this potato line since 1998 (EC, 2002).

1.1.4. Benefits of GM Crops

Genetic engineering of crops may lead to a general improvement in agriculture and food, health as well as the environment. Here given are some benefits of GM crops reported.

1.1.4.1. Increased Food Production

The world population is expected to reach 9 billion people by the year 2040, generating a 250 % increase in demand for food (Aluizio et al., 2003). Adding new land for crop production, reducing losses due to both biotic and abiotic stresses, and increasing crop productivity, are some of the measures that will increase food production. Measures to increase food production without having a serious impact on environment and natural resources are a limited option (NAS, 2000).

GM crops that are resistant to pests, diseases, and tolerant to herbicides, salinity, pH, temperature, frost, and drought have been developed (Taylor et al., 1997).
The bacterium *Bacillus thuringiensis* has been instrumental in developing insect resistant crops. Crystal Protein (Cry) genes have been transformed to a varieties of crops; corn, cotton, potato among others to control insects that cause food losses (Aluizio et al., 2003).

Besides Bt genes, viral genes have also been transformed in some major crops to combat viral infestation. The development of transgenic crops with inbuilt resistance to biotic stress would help to stablise annual food production. Cassava resistant to destructive cassava mosaic virus (Anon, 1996); rice resistant to rice yellow mottle virus have been developed (Pinto et al., 1999).

GM technology can also be used to convert major cereal crops that are annuals to perennials. This would reduce tillage and erosion, and lead to conservation of water and nutrients (Jackson, 1991). It would also increase crop yield during the year.

Increasing a crop's ability to withstand environmental stresses (e.g. extreme pH, salt, pests, heat, etc) will enable growers to farm in those parts of the world currently unsuitable for crop production. Drought resistance in GM crops for example will reduce water use in agriculture and thus be very useful in some tropical or arid regions where water is scarce. This will lead to increased global food production by

reducing crop loss and increasing yield, while conserving farmland and reduce pressure on irreplaceable natural resources like the rain forests (Uzogara, 2000).
Genetic modification can also lead to crops with enhanced nitrogen fixation that will reduce fertilizer use and cost of production (Laane, 1993). Increased food production through biotechnology will have a positive global impact by increasing the dietary staples (such as rice, wheat, corn, cassava, potatoes, bananas, beans, cereals, legumes, tubers) of many regions of the world (Uzogara, 2000).

1.1.4.2. Improved Food Quality

GM has led to improved shelf life and organoleptic quality in certain crops. For example the Flavr Savr GM tomato with a longer shelf life due to delayed ripening, softening, and rotting processes (Martina et al., 2000). Extending a product's shelf life will also benefit consumer to utilize the product for a longer time before it spoils. Such fruits and vegetables can better withstand handling, shipping, and storage. Farmers and consumers in developing countries where refrigeration is unreliable and expensive, and transportation a problem, they will benefit more from such fruits and vegetables (Thayer, 1999).

1.1.4.3. Improved Nutritional Quality

Vitamin A deficiency causes half a million children to become partially or totally blind each year (Conway et al., 1999). Traditional breeding methods have been unsuccessful in producing crops with a high vitamin A concentration and most national authorities rely on expensive and complicated supplementation programs to address the problem (NAS, 2000).
The transgenic rice ‘’Golden rice’’ with increased production of beta-carotene as a precursor to vitamin A has been developed (Ye et al., 2000). This rice variety will help to combat blindness resulting from a deficiency of vitamin A, a serious problem in less developed countries in Africa and South East Asia (Aluizio et al., 2003).

Cereal grains are deficient in essential micronutrients such as iron and Iron deficiency causes anemia in pregnant women and young children (Martina et al., 2000). About 400 million women of childbearing age suffer as a result, and they are more prone to stillborn or underweight children and to mortality at childbirth (NAS, 2000). Anemia has been identified as a contributing factor in over 20% of maternal deaths (after giving birth) in Asia and Africa (Conway 1999). Transgenic rice with elevated levels of iron has been produced using genes involved in the production of an iron binding protein and in the production of an enzyme that facilitates iron availability in the human diet (Goto et al., 1999).

Also GM foods with increased levels of naturally occurring anti-oxidant compounds (carotenoids, flavonoids, vitamins A, C, and E) have been developed. These compounds slow or shut down biological oxidation that appears to promote the development of some cancers and heart diseases (Smaglik, 1999; Philips, 1994).
Oil crops with reduced levels of saturated and Trans fatty acid content have been developed using GE (Liu et al., 1996). Such foods will reduce cholesterol levels in the body.

1.1.4.4. Improved Protein Quality

GE has improved protein quality of foods and feeds and there is less risk of allergies from GM foods than in conventional foods (such as Brazil nut and peanut) already in the market (De et al., 1997).

Improved protein quality may involve an increase in the essential amino acid content of the crop, such as methionine and lysine (Hauman, 1997). It may also involve improvement in the functional properties thereby expanding the use of plant protein in various food systems (Kitamura, 1995).

1.1.4.5. Improvement in Quantity and Quality of Animal Products

Transgenic animals will be tailored to produce more milk or meat with special qualities, for example, lactose-free milk, low fat milk, low cholesterol meats, low fat meats or meats with special protein and nutrient composition in a cost-effective process (Laane, 1993). Transgenic livestock can also be used to express large quantities of recombinant proteins such as fibrinogen in milk (Rohricht, 1999). Such Transgenic proteins will be good alternatives to blood proteins derived from donated human blood which is feared as a potential source of Human Immunodeficiency Virus (HIV) and Bovine Spongiform Encephalopathy (Uzogara, 2000).
Fish, which is a good source of dietary protein, could be conditioned to grow larger in a short period, thus becoming a viable option for aquaculture (Philips, 1994).

Experiments are underway for the developed of transgenic animals as a source of organs and tissues for transplanting. This is hoped to be a solution to the shortage of donor organs (Aluizio et al., 2003).

.

1.1.4.6. Health Benefits

Research is underway for the use of GM technology to produce vaccines and pharmaceuticals in plants. Some tropical crops such as banana, which are consumed raw when ripe, have been bio-engineered to produce proteins that may be used as vaccines against hepatitis, rabies, dysentery, cholera, diarrhea, malaria and other infections prevalent in developing countries (Anon, 1998).
These vaccines in edible foods will be beneficial to children in developing countries where such foods are grown and distributed at low cost, and where resources and medical infrastructure for vaccine production are lacking (Daie et al., 1993).

Some human genes have also been inserted into plant chromosomes to yield large quantities of experimental biopharmaceuticals. Tobacco and potato have been

engineered to produce human serum albumin (Uzogara, 2000). Oilseed rape and *Arabidopsis* have been engineered to yield the human neurotransmitter, Leu-enkephalin and monoclonal antibodies (Lesney, 1999). Work is also going on to produce insulin in plants. The insulin would be ingested by diabetics rather than received through shots (Uzogara, 2000).

About one-third of medicines used today are derived from plants, which produces them in minute concentrations. For example, the valuable anti-cancer agents vinblastine and vincristine are the only approved drugs for treatment of Hodgkin's lymphoma, yet are very expensive to produce (NAS, 2000). Current research is focusing on the use of GM technology to increase the yields of such active compounds, or to allow their production in other plants that are easier to manage than the periwinkle (Leech et al., 1998).

1.1.4.7. Environmental Benefits

Environmental benefits include reduced pesticides use by using insect resistant plants, reduction in the amount of land needed for agriculture, conservation of resources through use of less labor, fuel, fertilizer and water, water quality protection, and protection against plant diseases. Also some plants have been especially bio-engineered to enable them remove toxic waste from the environment (Bioremediation). Several researchers have reported encouraging results using plants to clean up the ravages of industries, agriculture, and petroleum production (Gray, 1998).

A project aiming to develop transgenic crop varieties for bioplastic production is underway (Aluizio et al., 2003). Such plastics from plants will be biodegradable and will reduce environmental pollution posed by plastics products from petroleum. Also scientists are developing an edible bioplastic that would allow foods to be cooked in their own packaging, thereby reducing the volume of domestic waste (Aluizio et al., 2003).

1.1.5. Potential Concerns of GM Foods

Potential concerns of GM foods/crops put forward by critics include the following:

1.1.5.1. Antibiotic Resistance

There is a concern that deliberately breeding antibiotic resistance into widely consumed crops may have unintended consequences for the environment as well as for humans and animals consuming the crops (Philips, 1994). According to a report from the British Medical Association, antibiotic resistance marker genes inserted into certain crops could be transferred to disease-causing microbes in the gut of humans or animals consuming GM foods. This could result in antibiotic resistant microbes in the population, and contribute to the growing public health problem of antibiotic resistance (Hileman, 1999).

1.1.5.2. Potential Toxicity

Genetic modification could inadvertently enhance natural plant toxins by switching on a gene that has both the desired effect and capacity to pump out a poison. Genes for some natural toxins such as protease inhibitors in legumes, cyanogens in cassava and lima beans, may be turned on and lead to an increase in levels of these toxins which can pose a hazard to the consumers of these crops (Uzogara, 2000).

1.1.5.3. Environmental Concerns

Environmentalists are concerned that transgenic crops will present environmental risks when they are widely cultivated (Kaiser, 1996). Genetically modified crops having herbicide and insect resistance could cross-pollinate with wild species, and unintentionally create hard-to-eradicate super-weeds especially in small farm fields surrounded by wild plants. These super-weeds can become invasive plants with potential to lower crop yields and disrupt natural ecosystems (Hileman, 1999).

Transgenic crops could also become weeds requiring expensive and environmentally dangerous chemical control programs (Rissler et al., 1993). Opponents of GM crops want regulations to demand proper studies to assess the risks of GM crops on the environment. They believe that Bt toxin, for example, can threaten beneficial insects by entering the food chain.

Plants engineered to contain virus particles as part of a strategy to enhance resistance could facilitate the creation of new viruses in the environment (Philips, 1994). Plants engineered to express potentially toxic substances such as drugs and pesticides will present risks to other organisms that are not intended as targets (Uzogara, 2000).

1.1.5.4. Potential Allergies

Genetic modification of food plants could transfer allergenic properties of the donor source into the recipient plant or animal**.** As well, genes from non-food sources and new gene combinations could trigger allergic reactions in some people, or exacerbate existing ones (Uzogara, 2000).
Pioneer Hi-bred International (a seed company now owned by Dupont) incorporated Brazil nut genes into soybeans to increase the protein content of its animal feed. This gene modification caused allergic reactions in consumers who were allergic to Brazil nut, so this product was voluntarily recalled (Nordlee et al., 1996).

1.1.5.5. Limited Access to Seeds through Patenting of GM Crops

Some critics of genetic modification argue that patenting which allows corporations to have monopoly control of genetically altered plants or animals violates the sanctity of life (Dickson, 1999). Critics also oppose the fact that seeds which have been largely known as commodity products are now regarded as proprietary products because of genetic modification. Many critics view the ‘terminator gene’ technology as a monopoly and anti-competition. Terminator gene technology

produces sterile seeds that will never germinate when planted (Koch, 1998). It forces farmers to buy new seeds each year from multinational companies so that farmers become dependent on the multinationals instead of sowing seeds from the previous years' harvest. It is argued that this would destroy traditional farming practices (Uzogara, 2000).

1.1.5.6. Threat to Crop Genetic Diversity

Critics of genetic modification of foods fear that commercialization of transgenic crops will pose a new threat to crop genetic diversity already endangered by current agricultural practices that favor the worldwide adoption of a few crop varieties (Philips, 1994). Genetic modification also reduces bio-diversity of the world's food supply through the use of ‘terminator’ seed technology, which produces sterile seeds, and controls seed supply especially in developing countries (Koch, 1998).

1.1.5.7. Religious, Cultural, and Ethical Concerns

Religious concerns are also voiced as some of the reasons for opposing genetic engineering of foods, while some people object to bio-engineered foods for personal, ethical, cultural, and esthetic reasons, as well as infringement on consumer choice, and inability to distinguish GM foods from non-GM counterparts. For example, Jews and Muslims will object to grains that contain pig genes, and usually insist on Kosher and Halal foods whose purity can be documented. Vegetarians may similarly object to vegetables and fruits that contain no animal genes (Crist, 1996). Some people fear eating plant foods containing human genes (Uzogara, 2000).

1.1.5.8. Concerns of Animal Rights Groups and Organic Farmers

Animal rights groups strongly oppose any form of cloning or genetic engineering involving animals, or use of animals in research, and have sometimes resorted to vandalizing animal research facilities (Kaiser, 1999).

Organic farmers fear that GM foods would obscure organic foods because of lack of labeling, and they feel that the biotechnology revolution could make it difficult for people to locate non-GM crops. There is a concern that organic crops might be contaminated through cross breeding of herbicide resistant plants with wild relatives, or through cross pollination with GM crops in neighboring farms, thereby creating ‘monster weeds’ resistant to natural pesticides normally used by organic farmers (Uzogara, 2000). There is also a fear that pests resistant to Bt toxin will be produced (Koch, 1998).

1.1.5.9. Fear of the Unknown

Consumers also have a genuine ‘fear of the unknown’ in that deadly microorganisms or super plants might be released during field testing or field trials, and accidents in biotechnology laboratories might lead to release of toxic agents, poisons, or biological toxins which will threaten human and animal populations (Uzogara, 2000). Alliance for Bio-ethics, The Pure Food Campaign, the Green Peace Movement, among others accuse the regulatory Authorities especially in US, for robbing consumers of the right to know what is in their food. They maintain that the science of genetic engineering is relatively young, less than 50 years old, and nobody knows the consequences of these genetic alterations in the future (Uzogara, 2000).

1.2. Detection of Genetically Modified Organisms

The need to monitor and verify the presence and the amount of GMOs in agricultural crops and in products derived thereof has generated a demand for analytical methods capable of detecting, identifying and quantifying either the DNA introduced or the protein(s) expressed in transgenic plants, because these components are considered as the fundamental constituents (Gadani et al., 2000; Hemmer, 1997; Lüthy, 1999; Meyer, 1999).

The development and application of reliable detection and quantitative analytical methods is essential for the implementation of labelling rules especially in countries where GM labeling is mandatory.

In general, detection for the presence of GMO consists of three different steps (Anklam et al., 2002):

1. Screening of GMOs in order to gain a first insight into the composition of the food and agricultural product. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results.

2. Identification to reveal how many GMOs are present, and if so, whether they are authorized within the country's regulations. A prerequisite for the identification of GMOs is the availability of detailed information on their molecular make-up. Molecular registers that, along with the scientific data, contain the tools for control authorities to design appropriate identification methods, are essential to fulfill this task.

3. Quantification, in order to determine the amount of one or more authorized GMOs in a product or seed lot, and to assess compliance with the threshold regulation. The threshold value for GMO labeling varies in different countries. In EU, the threshold of 0.9 % was established for the adventitious presence of (authorized) genetically modified material in food and food ingredients in respect of labeling under Commission Regulation Number 49/2000, (http://www.efsa.eu.int/science/gmo, 15/01/2005). Where as in Japan, threshold is 5%, (http://www2.ipcku.kansai-u.ac.jp/~kshigeru/res/GMO.pdf, 15/01/2005).

Some of important steps in GMO detection process are sampling and sample preparation (Anklam et al., 2002). The sampling procedure determines how representative the results will be, whereas quality and quantity of analytes may vary depending on the sample preparation.

Several methods have been developed either based on DNA detection, or on protein detection. The methods vary in their reliability, robustness and reproducibility; in combination with different levels of cost, complexity, and speed, and there is no one method that is applicable to all circumstances (Anklam et al., 2002).

1.2.1. DNA based methods

Among DNA analysis methods, PCR in its different formats has been the most widely used method for GMO detection/analysis and is a generally accepted method for regulatory compliance purposes (Anklam et al., 2000).

The PCR allows the million-fold amplification of a target DNA fragment in a highly sensitive and specific manner. The target sequence in each reaction cycle is duplicated and the number of target sequences grows exponentially in the consecutive cycles according to the number of cycles.

Confirmation of the identity of a PCR amplicon is a necessary in order to ensure that the amplified DNA product actually corresponds to the chosen target sequence and is not a product of non-specific binding of the primers. Several methods are available for this purpose (Anklam et al., 2002).
The simplest approach is to control whether the PCR products have the expected size is by gel electrophoresis. However, there is a risk that an artifact of the same size as the target sequence has been amplified. Therefore, the PCR product should at least be additionally verified for its restriction endonuclease profile (Meyer, 1995).

Verification can also be done by a Southern blot assay, nested PCR and/or sequencing. For southern blotting assay, the amplicon is separated by gel electrophoresis, transferred onto a membrane and hybridized to a specific DNA probe (Anklam et al., 2002)
Nested PCR, the PCR product is re-amplified using another primer pair, located in the inner region of the original target sequence (Jaccuad et al., 2003). The most

reliable way to confirm the authenticity of a PCR product is its sequencing (DMIF-GEN, 1999).

Any PCR-based detection strategy depends on a detailed knowledge of the transgenic DNA sequences and of the molecular structure of the GMOs in order to select the appropriate oligonucleotide primers. For routine screening purposes, Genetic control elements such as the cauliflower mosaic virus (CaMV) 35S promoter (*P-35S*) and *Agrobacterium tumefaciens nos* terminator and neomycin phosphotransferase (nptII) selection marker are used. These genetic control elements are present in many GMOs currently on the market (Hemmer, 1997).

Some GMOs have been approved, in which more tissue- and stage-specific as well as non-heterologous regulatory genetic elements have been introduced (Wurz, 1999) and also in the future selection markers will be avoided (Anklam et al., 2002). Additional target sequences therefore will be needed in order to guarantee complete screening procedures.

Also the choice of the primers used should allow detection of as many variants as possible of a GMO marker. For example, there are at least eight variants of *P-35S* used in GM crops (Hemmer, 1997). At the screening step, the detection of these GMO markers is only an indication that the analysed sample contains DNA from a GM plant, but does not provide information on the specific trait that has been engineered in the plant (Anklam et al., 2002).

Target sequences that may occur as natural contaminants in the sample should be avoided (Anklam et al., 2002), i.e. DNA from plant viruses and bacteria, because of the risk of false positives. Therefore, a sample with a positive signal in P-35S/nos3' screening should be analysed for naturally occurring CaMV and *Agrobacterium tumefaciens* infection, respectively (Wolf et al., 2000).
However, it should be considered that the host range of the CaMV is restricted to cruciferous plants such as oilseed rape, and that the nos3' terminator sequence is

found only in certain strains of *A. tumefaciens*, which are pathogenic to certain crop species (Anklam et al., 2002).
As to the *A. tumefaciens* frequently found in soil, it is generally not virulent, i.e. it does not carry the Ti-plasmid with the T-DNA and the oncogenes. Thus, the nos3' gene and its control elements are not present in these naturally occurring strains (Anklam et al., 2002).

PCR based techniques that can be used for quantification of GM material in sample have been reported. Quantitative competitive PCR (QC-PCR) and real-time PCR, establish a relationship between the concentration of target DNA and the amount of PCR product generated by the amplification. A detailed discussion of quantitative PCR approaches is described by Ferré (1998).

Another strategy for GMO identification recently discussed makes use of amplified fragment length polymorphism (AFLP), a DNA fingerprinting method, which has already been used successfully to discriminate between and identify plant varieties (Preston et al., 1999). Recent experimental findings indicated that the AFLP technology could be adapted for the detection of genetic modifications by using a GMO-specific primer in conjunction with a primer specific for the surrounding genomic region (Windels et al., 1999).

Alternative techniques for GMO analysis include: Chromatography, Near infrared spectroscopy and DNA chips.
Chromatography is used where the composition of GMO ingredients such as fatty acids or triglycerides is altered, to detect the differences in the chemical profile (Byrdwell et al., 1996). Near infrared spectroscopy (NIR) is used where genetic modifications alter the fiber structure in plants, whereas no significant differences could be observed in the content of protein and oil (Hurburgh et al., 2000).

To cope up with rapid development of GM plants, new technologies and instruments will be needed for the high throughput and low cost detection of an increasing

variety of genes (Anklam et al., 2002). New technologies such as micro-arrays and micro-fluidic systems (Sanders et al., 2000) appear to be a promising area for GMO analysis applications. Although several authors have reported on PCR micro-systems of different complexities (Ibrahim et al., 1998; Waters et al., 1998), few examples of microchip applications to GMO analysis have been described so far (AOAC, 2000).

Although biotic resistant transgenic potato cultivars were approved for commercial cultivation since 1996, there are still a few studies published on the detection of GM potatoes compared to maize and Soybeans. The studies on GM potato detection so far reported include the following:

Jaccaud et al (2003) investigated the qualitative PCR methods for the screening and identification of insect and viral resistance in GM potatoes. An elaborate of a number of primer sets targeting the foreign genes in Newleaf, Newleaf Plus and Newleaf Y lines in raw and processed form is given in their study.

Donna et al (2004) developed a method for the detection of synthetic Cry 3A in transgenic potatoes using PCR. The synthetic Cry 3A was detected in six different transgenic lines as well as in processed food products such as potato chips and frozen French fries.

Jae et al (2004) developed qualitative and quantitative PCR for the detection of GM potatoes. Specific primers to allow identify each line of Newleaf, Newleaf Y, and Newleaf Plus GM potatoes are reported. Besides, Multiplex PCR method for the simple and rapid detection of the three lines of GM potato in mixture sample was also established.

1.2.2. Protein based Methods

Detection of a novel protein synthesized by a gene introduced during transformation constitutes an alternative approach for the identification of genetically modified

plants. Protein detection methods are based mainly on immunoassays (antigen and antibody) and since they require proteins with an intact tertiary or quaternary structure, these methods are limited to fresh and unprocessed foods (Bonfini et al., 2001).

The crucial component of an immunoassay is an antibody with high specificity for the target protein (antigen). Immunoassays can be highly specific and samples often need only a simple preparation before being analysed. Moreover, immunoassays can be used qualitatively or quantitatively over a wide range of concentrations

A valid identification of the foreign protein in GMOs using immunoassays depends on the availability of the particular proteins for development of the antibodies; desired affinity and specificity of antigen/antibody, hydrophobicity and the tertiary structure of antigen are also very important factors (Anklam et al., 2002).

The most common type of immunoassay is the Enzyme-Linked ImmunoSorbent Assay (ELISA), which utilizes an enzyme-labeled immunoreactant (antigen or antibody) and an immunosorbent (antigen or antibody bound to a solid support).

Bonfini et al (2001) describes more details of GMO detection by protein-based methods.

Table 1.3. DNA and protein based methods compared

DNA Based Methods	**Protein Based Methods**
Highly sensitive	low sensitivity (1% of GM protein)
Work with processed & unprocessed products	Not suitable for processed products
Multiple GM varieties tests simultaneously	Limited to small no. Of varieties
Highly skilled personnel required	Less trained personnel
Takes no. Of days to perform (3-5 days)	Rapid turnover (5-20 mins; 24 hrs)
More expensive	Relatively cheap

1.3. Regulation of Foods Derived from GMOs

Policies, laws and guidelines for the assessment of food safety continually evolve over time. The evolution in individual jurisdiction is affected by both science and society (König et al., 2004). Scientific advances improve our understanding of health implications of foods whereas changing societal values can lead to shifts in emphasis in consumer protection policies and regulatory and institutional change.
There are two types of regulatory frameworks for foods derived from GM crops; Process based legislation adopted in European Union (EU) and Australia, and product based legislation in USA and Canada (König et al., 2004).

1.3.1. Regulations in EU

The EU legislation on GMO was instituted in the early 1990s. Regulatory framework is ‘’process based’’ also called ’’horizontal’’, and it includes the following directives:
Directive 2001/18/EC on experimental releases and the marketing of GMO in EU. It establishes a step by step approval process for a case by case assessment of the risks to humans health and the environment prior to authorizing the placing on the market or release into the environment of any GMO or product containing GMOs (European Commission, 2001).

Directive 258/97/EC on novel foods and food ingredients regulates the authorization and labeling of novel foods including food products containing, consisting of or produced from GMOs. Novel foods regulation requires the risk assessment and pre-market approval of novel foods, and also specifies labeling requirements for certain categories of novel foods. This regulation gave the EC a clear role in the governance of food safety in the EU (European Commission, 1997).

Regulation 178/2002/EC on the general principles of food law and the establishment of the European food safety Authority (European Commission, 2002a).

Regulation 50/2002/EC on labeling of genetically modified additives and flavorings. There have been recent changes in regulations:

Regulation 1830/2003/EC on traceability and labeling of GMO and traceability of food and feed produced from GMO and amending directive 2001/18/EC (European Commission, 2003a).

Regulation 1829/2003/EC on GM food and feed require the traceability of GMOs through out the food chain from farm to table and provide consumers and farmers with information by labeling all food and feed consisting, containing or produced from GMOs (European Commission, 2003b).

Regulation 65/2004/EC of January 2004, establishing a system for the development and assignment of unique identifiers of GMO.

1.3.2. Regulation in US

US regulatory framework for GM crops was laid out in 1986 'coordinated framework for regulation of biotechnology' (US. OSTP, 1986). US regulation on GMO is a vertical, ''product based'' framework. United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and Food and Drug Authority (FDA) are agencies concerned with GMO regulation in USA (König et al., 2004).

USDA regulates the import, interstate movement, field trials release and commercial release of GM crops under the Federal plant pest Act and the Plant Quarantine Act, which are administered by the Animal and Plant Health Inspection service (APHIS).

FDA is authorized with human food and animal feed safety and the wholesomeness of all plant products including those produced via genetic modification.

EPA has regulatory oversight for all GM crops that produce a plant pesticide. Unlike EU, GM labeling is not mandatory in US, except for foods that present a health risk to subgroups of the population.

1.3.3. Regulation in Canada

All plants with novel traits are regulated, regardless of whether a plant with novel traits was produced by conventional breeding or recombinant DNA techniques (CFIA, 1998). Foods derived from GM crops are considered as novel foods under the Food and Drug Act (CFIA, 1998). The Canadian Biotechnology Advisory Committee recently reviewed the GM regulation; its recommendations include that research be carried out in order to monitor for hypothetical long-term health effects (CBAC, 2002).

1.3.4. Regulation in Japan

In Japan, Regulation of GMOs is governed by the Ministry of Agriculture, Food and Fisheries (MAFF) and the Ministry of Health, Labor, and Welfare (MHLW). It includes safety of GM crops and other food additives that contain organisms or have been obtained through recombinant DNA techniques (Japan MHLW, 2000).

1.3.5. Regulation in Turkey

Cartagena biosafety protocol, which seeks to protect biological diversity from the potential risks posed by modern biotechnology, was signed in 2000. The food law (No 5179) that focuses on controlling all commercialized foods was implemented in May 2004. A draft of national biosafety law has been formulated and given one

month for public reactions or views, after which it will be implemented (www.tagem.gov.tr, 10/09/2004).

1.3.6. Regulation in Australia

In Australia and New Zealand, the food standards Australia New Zealand (FSANZ) has regulatory oversight over food safety, for foods derived from GMOs (FSANZ, 2001). Part of regulation addresses marketing of foods and post market monitoring requirements (FSANZ, 2000).

1.3.7. Regulation in South Africa

South Africa is the leading country regarding adoption GM crops in Africa. GMO legislation was inacted in 1997 ″ GMOs Act, 15 of 1997′, and came into effect on 1 December, 1999. It establishes the following organs with their responsibilities (Jennifer , 2000)

The Executive Council: The national decision-making structure that will approve or deny permits for all stages of GMO development and release. Composed of government departments and a scientific advisor, who is the chairperson of the Scientific Advisory Committee.The Scientific Advisory Committee; advises the Executive Council on the biosafety of applications submitted for permits. The Registrar for the GMO Act in the Registrar's office, who administers the act. The registrar receives applications and issues permits on the instruction of the Executive Council. The act makes allowance for inspection, confidentiality, appeals and regulations.

Although the Regulatory frameworks differ across jurisdictions, the approaches to the safety assessment of foods derived from GM crops are similar in most countries, as they are based on general principles for risk analysis and international guidelines

for the safety assessment of foods derived from genetically modified organisms (König et al., 2004).

Table 1.4. Approval status of GM potatoes

Event	Trait	Company	Country approved
Newleaf ATBT04-6, ATBT04-30, ATBT04-36, ATBT 04-31	Insect resistance	Monsanto	Australia, Canada, USA
Newleaf BT6, BT10, BT12, BT16, BT17, BT18, BT23	Insect resistance	Monsanto	Australia, Canada, Japan, USA
Newleaf Y RBMT15-101, SEMT15-02, SEMT15-15, HLMT15-46	Insect & PVY resistance	Monsanto	Australia, Canada, USA
Newleaf Plus RBMT21-129, RBMT21-152, RBMT21-350	Insect & PLRV resistance	Monsanto	Australia, Canada, Japan, USA
Newleaf Plus RBMT22-082, RBMT22-186, RBMT22-238, RBMT22-262	Insect & PLRV resistance	Monsanto	Australia, Canada, Japan, USA
Newleaf SPBT02-5, SPBT02-7	Insect resistance	Monsanto	Australia, Canada, Japan, Romania, USA

Source: BATS, 2003.

1.4. Safety Assessment of Foods Derived from GM Crops

Safety assessment of foods derived from GM crops is the same as those for conventional or other types of novel foods (Cockburn, 2002). The objective is to determine whether these GM foods are as safe as foods produced from conventional crops. Concepts for the safety assessment of GM crops focus on the new gene products and whole foods derived from the GM crop, and any functional and chemical changes that result from the genetic modification. The assessment of foods derived from GM crop, relies on the use of a food generally recognized as safe as a comparator (FAO/WHO, 1991).

Both intended and potential unintended effects from genetic modification are taken into account.

The assessment involves the following steps (König et al., 2004):

i). Characterization of the parent crop; ii) characterization of the donor organism(s) from which any recombinant DNA sequences are derived, the transformation process, and introduced recombinant DNA sequences; iii) safety assessment of introduced gene products (proteins and metabolites); iv) food safety assessment of whole food derived from or edible part of GM crop.

Methods for the detection of unexpected changes in the composition due to the genetic modification process are discussed and evaluated by Cellin et al (2004). Possible consequences of transfer of the recombinant sequences to gastrointestinal microflora or to humans should be assesses and are evaluated in the paper by Van den Eede et al (2004).

For the food safety assessment of whole foods derived from or edible part of GM crops, the concept of 'substantial equivalence' is used.

1.4.1. Substantial Equivalence

It's a comparative assessment approach. Its requires the comparison of the GM crop and an appropriate safe comparator according to the agronomical and morphological

characteristics, and the chemical composition, including macro- and micro-nutrients, key toxins and key anti- nutrients (König et al., 2004). Comparator is usually the traditional bred parent crop, and substantial equivalence concept allows identification of significant differences between the GM crop and comparator (OECD, 1993).

The successful application of the concept of substantial equivalence will largely depend on: the availability of an appropriate comparator and an understanding of the range of variation to be expected within the measured characteristics of that comparator; the choice of parameters; and the ability to discriminate between differences in the GM crop and comparator that result from the genetic modification and those differences in plant's germplasm, some of which may be attributed to somaclonal variation introduced during tissue culture (König et al., 2004).

The concept of substantial equivalence is a starting point and guiding concept for safety assessment, not its conclusion (FAO/WHO, 2000 and codex Alimenatrius Commission, 2003). It's widely accepted by international and national agencies as the best available guidance for the safety assessment of new GM crops. As with all scientific concepts, the concept of substantial equivalence is evolving and, together with guidelines, making its application more systematic (König et al., 2004). Dialogue between experts and civil society will contribute over time to further and structure risk analysis strategies to improve the salience of assessment to address concerns of policy markers and the public (Jasanoff, 2000).

1.5. Impact of GM Crops on the Third World

One of the biggest problems inflicting third world is hunger or ability to feed themselves. FAO estimates that 800 million people have insufficient food to meet their basic needs, while 40% of the world's entire population suffers from malnutrition (FAO/UNICEF, 1994).

Transgenic crops are developed amidst promises that they will help the third world feed itself. However, critics argue that increasing the amount of food on planet is not necessarily the solution to feeding the hungry. Rather fighting poverty, created by complex mix of social and political factors (Nottingham, 2003).

Multinational biotechnology companies have shown little interest in foods grown highly in third world such as millet, cassava or yams, instead they have concentrated on developing crops that will earn high profits (Nottingham, 2003). The claims that they are making important contributions to world food supplies are not at present justified. Many countries in thirds world posses the genetic resources to generate a sustainable food supply (Nottingham, 2003).

From the economic point of view, farmers in third world may also find their markets shrinking due to competition from alternatives produced in industrialized countries using genetic engineering strategies. The advantage of land and appropriate climate are no longer key factors in the production of oils, flavorings, sweeteners and other products from tropical crops. Developed countries with biotechnology advancement will soon corner new agriculture markets, while many developing countries will suffer massive loss of export earnings. The technology as it currently stands is not reducing the gap between the rich and poor (Nottingham, 2003).

Farmers in developing countries will hope to increase their incomes by growing transgenic crops. However they will have to pay for transgenic seeds together with other farm inputs e.g. herbicides, fertilizers and many small-scale farmers can't afford them.

Far from helping feed the hungry, the new agriculture technology may be increasing the economic problems of developing countries, exacerbating poverty and malnutrition. If the technology can be adapted to the specific needs of developing countries, some transgenic crops might come to make positive contribution to food production (Nottingham, 2003).

1.6. Consumers' Awareness and Perception of GMOs

Although modern Biotechnology presents a range of potential benefits in agriculture, environment as well as in health sector, some consumers are still skeptical about it and it has attracted intense public and political debate.
Differences in perception exist between different countries, between different individuals within countries, and within different individuals at different times and within different contexts (Burger et al., 2001).

Resistance to the application of genetic modification is not universal, consumer attitudes towards the technology depend both on the area of application and on the type of modification (IFIC, 2000).

There have been several surveys on public perceptions of biotechnology (NSF, 2000; Angus Reid, 2000; IFIC, 2000; Hoban, 1996; Berrier, 1987; FMI, 1995; Russell et al., 1987). These surveys have shown that people's attitudes vary towards GMO and are driven by a number of inter-related factors such as knowledge level, awareness of benefits, confidence and trust (Hoban, 1996).

There is greater acceptance of medical applications (particularly those leading to development of medicines and vaccines) than there is for food biotechnology products (Klaus et al., 2001).

In 1996 survey of the European public's attitudes towards biotechnology under the title of Eurobarometer clearly showed that some applications of biotechnology are well accepted, although the factual knowledge about basic biology is remarkably low (Braun, 1999). Biotechnology in basic research and in the medical field is well accepted: this holds for pharmaceuticals, diagnostics and for vaccines (Braun, 1999).

The Angus Reid World Poll conducted in 8 countries (Australia, Brazil, Canada, France, Germany, Japan, United Kingdom and the United States) during late

November and early December of 1999 (Angus Reid, 1999a) examined public awareness and perceptions regarding genetically modified foods. The results showed that to a large extent the public is aware of genetically modified foods.

In 2000, The Angus Reid World Poll surveyed 5, 000 adult consumers on genetically modified foods in Australia, Brazil, Canada, France, Germany, Japan, United Kingdom, and United States (Angus Reid, 2000). Results from this international survey indicate that consumers around the world have a negative view about genetically modified foods and perceive the issue as one where the risks outweigh the benefits.
According to the Angus Reid World Poll (Angus Reid, 2000), when consumers were asked about perceived risks associated with genetically modified foods, 31 per cent of consumers cited food safety and health concerns. An equal proportion (30 %) also said that they were concerned about the unknown impact and experimental nature of GM foods.

However many people in the United States and Japan believe that they are sufficiently informed about the new technology and GM foods, and accept such foods without worries as long as the regulatory agencies give scientific assurance for the safety, wholesomeness and nutritional quality of the foods (Hoban, 1999). There is a concern however that continued opposition of GM foods abroad may soon influence acceptability of GM food in the United States

1.7. Objectives of the study

The main objective of this study was to investigate if genetically modified potatoes exist on Turkey's food market.

The other objective was to apply, test and develop a method for the detection of transformed genetic elements in potato samples obtained randomly on the market. This study is also aimed to guide GMO detection laboratories, which will be established and accredited in Turkey in the near future.

CHAPTER 2

MATERIALS AND METHODS

2.1. Potato Materials

Potato samples were obtained randomly from different markets both bazaar and supermarkets in Ankara, Izmir Istanbul, Afyon and Eskeshir. Samples were obtained between January and February of 2004 and in November of the same year. Additionally some potato samples were obtained from USA, Belgium and Tanzania for comparative study. A total of 30 samples of raw potato tubers were collected (Table 2.1).

2.2. Reference Materials

The isolated DNA of Transgenic potato (Newleaf Russent Burbank) was kindly provided by Prof. Dr Douches Love of Michigan State University (Crop Science Dept), USA. The DNA was extracted from the leaves of the transgenic potato using the CTAB method. The non-transgenic potatoes were obtained from Cappadocia region.

Bacillus thuringiensis biovar *tenebrionis* (BGSC No: 4AA1) for extraction of natural cry3A was kindly provided by Bacillus Genetic Stock Center, The Ohio State University, USA.

2.3. Chemicals, Buffers and Solutions

Chemicals used together with their suppliers are listed in Appendix A. The composition and preparation of buffers and solutions are given in Appendix B.

Table 2.1. Potato samples used in the study

Number	Sample code	Market place	Number	Sample code	Market place
1	G1	Ankara (U)	17	14	Ankara (B)
2	H1	Ankara (S)	18	13	Ankara (U)
3	H2	Ankara (U)	19	B1	Ankara (B)
4	R	Ankara (U)	20	T2	İstanbul (U)
5	T1	Ankara (S)	21	U1	İstanbul (U)
6	X1	Ankara (S)	22	K1	Eşkshehir (U)
7	Y1	Ankara (B)	23	R1	Ankara (S)
8	M2	Ankara (B)	24	A2	Ankara (U)
9	M3	Ankara (B)	25	S	Ankara (B)
10	F1	Afyon (U)	26	S1	Ankara (S)
11	Z2	İzmir (U)	27	B	Ankara (B)
12	Z1	İzmir (U)	28	C3	USA (U)
13	D	Ankara (U)	29	L	Belium (U)
14	P1	Ankara (S)	30	Tz	Tanzania (U)
15	N1	Ankara (S)	31	15 (TR)	USA
16	4	Ankara (B)	32	Q (Non-TR)	Cappadocia

Key: B, bazaar; S, supermarket; U, unknown;

Sample coded 15 was transgenic and sample coded Q, non-transgenic.

2.4. DNA Extraction

2.4.1. Potato DNA extraction

Each tuber was washed in running water to remove adhering soil particles. The tuber was cut into 2 halves with sterile knife, from one half; an inner cube was obtained, without the outer skin layer. The inner cube was washed again in running water before cutting it into small slices. The slices were ground in mortal using liquid nitrogen to get a homogenous white powder, which were stored in $-80^{O}C$ pending further analysis.

DNA was extracted from potato powders using the CTAB method as described by Wuff et al (2002) with some modifications.

Using sterile spatula, 1g of homogenized powder was transferred to a tube containing 5 mL of extraction buffer [100 mM Tris (pH 8), 20 mM EDTA (pH 8), 2% CTAB, and 2.5 M NaCl] at 65°C.
To the suspension, 100 mg of PVP (polyvinylpyrrolidone, Sigma 40000) was added, and then mixed by inversion. The tubes were incubated at 65°C in a water bath for 60 min with occasionally shaking at 15 min interval.
The tubes were removed from the water bath, cooled to room temperature, and to the suspension 5 mL of chloroform-isoamylalcohol (24:1) was added. The tubes were mixed by inversion until an emulsion formed.

Samples were then centrifuged at 5000 rpm (2500 *g*) for 20 min and supernatant transferred to a new tube. An equal volume of chloroform-isoamylalcohol (24:1) was added to the supernatant, mixed well, and centrifuge for 20 min at 10,000 rpm (8160 *g*).

To the supernatant, an equal volume of ice-cold isopropanol was added and slowly inverted. After incubating on ice for at least 20 min, samples were centrifuged for 20 min at 5000 rpm (2500 *g*) to collect the DNA pellet.
The supernatant was poured off, the pellet washed by suspending in 1 mL of 70% ethanol; centrifuged for 5 min at 7000 rpm (4000 *g*). The supernatant was poured off; pellet dissolved in 400 µl of TE, and equal volume of chloroform – isoamylalcohol added.

Mixed suspension was centrifuged for 20 min at 14000 rpm (16000 g), supernatant transferred to a new tube and 2 volumes of 100% cold ethanol added. Mixture was incubated at -20^{0}C for 20 min, after which centrifuged for 20 min at 16000 g to collect the pellet. The pellet was washed in 70% alcohol then dried in a flow cabinet, before resuspension in 100 µL of double distilled water. RNA was removed

by adding 1µl of RNAase A (Fermentas #EN0531) then incubated at 37°C for 30 min. All isolated DNA was stored at -20^0C until further analysis.

2.4.2. *Bacillus thuringiensis biovar tenebrionis* chromosomal DNA isolation

A standard procedure devised for bacillus species (Cutting and Horn, 1990) was used. 1.5 ml of overnight culture is centrifuged at 13000 rpm for 5 min. The pellet is resuspended in 567 µl TE buffer by pipetting. 30 µl proteinase K (20 mg/ml), 6 µl RNase A (10 mg/ml), 24 µl lysozyme (100 mg/ml) and 30 µl 10% SDS are added and mixture incubated at 37^0C for 1 hour. After the incubation, 100µl of 5 M NaCl was added and mixed, and then 800µl of CTAB/NaCl solution added and mixed. The mixture is incubated at 65^0C for 10 min. The mixture was extracted with the same volume of phenol/choloroform/isoamylalcohol, and then centrifuged at 13000 rpm for 10 min. The upper phase was transferred to a new tube and 0.7 volume of isopropanol added, mixed and centrifuged for 15 min. The supernatant was removed and the pellet washed with 1 ml of 70% alcohol by centrifuging for 5 min. The pellet was dried at 37^0C for 1 hr then dissolved it in 20µl TE buffer.

2.5. Determining the DNA Purity

The purity of isolated DNA was determined by measuring its Absorbance (A) using U.V spectrophotometer. The UV spectrophotometer was set to 260 nm. Following the operating procedures, 1 ml of double distilled water in a quartz cuvette (1 cm) was used as "blank" to provide a zero reading. In the second quartz cuvette, DNA sample was diluted by adding 20 µl of the DNA solution to 980µl of double distilled water and mixed by inversion for 30 sec. Absorbance at 260 nm was measured and recorded, and same steps were repeated for measuring absorbance at 280 nm.
The ratio A_{260}/A_{280} was calculated and used to estimate the purity of DNA.

The DNA concentrations were worked out by estimation on agarose gel using known concentrations of phage lambda DNA digested by *PstI* and *Hind III* enzymes separately.

Besides DNA purity measurements using UV spectrophotometer, isolated DNAs were run on 0,8% agarose to check its resolution, molecular weight and whether it was intact.

2.6. Agarose Gel Electrophoresis

Electrophoretic Gel system used was that of Thermo Electron Corporation, USA. The gel casting tray was sealed by rubber tabs and comb properly positioned. The 0.8% and 2% agarose was used for DNA isolates and PCR products respectively. The gel was prepared by adding appropriate amount of agarose in 1X TAE Buffer (Appendix B). Agarose was melted by heating in microwave oven for 2-3 minutes with interrupted swirling to dissolve the agarose. To 30 ml of melted gel at 50 - 60^0C, 1 µl of Ethidium Bromide stock solution was added, mixed and the agarose solution poured in gel tray. Upon solidifying, the gel was put in electrophoresis tank containing 1X TAE buffer covering the gel wells. Samples (7 – 10 µl) were loaded into individual wells together with size markers *Pst*I digested lambda DNA or 100 bp DNA ladder Plus (Fermentas # SM0321). 3 – 5 µl of size marker was used and electrophoresis was run at 80 Volt for 50- 60 minutes.

Separated DNA bands were detected by illumination of the gel with UV radiation at 260nm and photograph taken using Nikon camera.

The *Pst* cut molecular weight marker was used for calculating molecular weights of DNA fragments after electrophoretic separation. Lambda DNA (Fermentas, cat.# SD0011) was digested with PstI (Fermentas # ER0611) restriction endonuclease. To 8 µl of double distilled water, 7 µl of Buffer, 5 µl of PstI, and 50µl of lambda DNA were added and mixture incubated for 2 hrs at 37^OC.

2.7. Primer Design

Primer set Kn- F and Kn- R was used to detect *E.coli* Transposon 5-neomycin phosphotransferase (nptII) gene. Primer sequences used for the detection of nptII gene were obtained from study by Sönmezalp (2004).

Primer set 3A-F/3A-R used to detect synthetic Cry 3A gene was designed in our laboratory using Primer 3 software programme (www-genome.wi.mit.edu/cgi-bin/primer/primer3, 18/06/2004). Cry3A gene sequences were obtained from Genbanks. The cry3 gene transformed in transgenic potato lines is synthetic and its homology with natural cry3A gene from *Bacillus thuringiensis tenebrionis* was compared. Primer sequences were selected from synthetic cry3A regions that were not completely homologous to natural cry3A sequences. This was aimed to avoid false positives i.e. one primer set amplifying both synthetic and natural Cry3A gene.

Primer sequences used for the amplification of natural cry3A gene were obtained from study by Donna et al (2004). Primer sets Pat-F and Pat-R, 35S-F and 35S-R, and Nos-F and Nos-R were used to detect patatin gene, 35S-Promoter Nos terminator respectively. The primer sequences were obtained from previous studies, (footnote) Table 2.2.

All Primers were synthesized by Iontek (Istanbul, Turkey) and always stored at – 20^0C.

Table 2.2. List of primers used in the study

Target	Primer	Sequence (5'-3')	Position	Target size (bp)	Accession No
NptII gene[a]	Kn-F	ttgctcctgccgagaaag	528-545	459	AF274974
	Kn-R	gaaggcgatagaaggcga	986-1003		
			2771-2789	124	X03932
Patatin gene[b]	Pat-F	ctcattaggcactggcact	2873-2894		
	Pat-R	gtaagaacttgctgcactagtc			
			1241-1232	195	AF078810
35S-P[b]	35S-F	gctcctacaaatgccatca	1389-1408		
	35S-R	gatagtgggattgtgcgtca			
			39-58	180	U12540
			198-218		
Nos-T[c]	Nos-F	gaatcctgttgccggtcttg			
	Nos-R	ttatcctagtttgcgcgcta	60-79	479	X70979
			518-538		
Synthetic cry	3A-F	gaagggtatctccgttgtgg	294-311	550	J02978
3A	3A-R	cagcaatgtcctctttctcgt	843-826		
Natural cry3A[c]	NC3A-F	tgaggtgccacctaacca			
	NC3A-R	ggcagcttgtgcatatgt			

[a] Sönmezalp, 2004; [b] Jaccaud et al., 2003; [c] Donna et al., 2004

2.8. Polymerase Chain Reaction (PCR)

Two – five microliters of the isolated potato DNA was added to reaction mixture and final volume of 30 µl was used in all PCR assays. The reaction mixture contained double distilled water, 1X PCR buffer, $MgCl_2$, dNTP mix (Fermentas # R0191), primer pair and Taq DNA polymerase(Fermentas # EP0405).

For each PCR set, a reagent (negative) control was always included to check reagent contamination. In the negative control tube, no template DNA was added, instead double distilled H_20 was added to fill up 30µl reaction volume. Heating was done in 0,2 ml thin wall PCR tubes (Axygen, USA), using Thermal cycler TC-412 (TECHNE, USA).

2.8.1. Patatin Specific PCR

In order to avoid false negatives, Patatin specific PCR was conducted to determine if isolated potato DNA was amplifiable. Primers sets Pat- F/Pat- R were used to target patatin protein, a major storage protein in potatoes. The concentrations of reaction components used are shown in Table 2.3.

Table 2.3. Concentrations of PCR components for Patatin gene

PCR components	Final concentrations
Sterile ddH20	19.6 µl
10X PCR buffer	3.0 µl (1X)
25 mM $MgCl^2$	1.8 µl (2.5mM)
10 mM dNTP mix	0.6 µl (0.2mM)
Pat-F	0.4 µl (0.5µM)
Pat-R	0.4 µl (0.5µM)
Taq DNA polymerase	0.2 µl

After preparation and assembling of PCR reaction mixture, four micro liters of DNA was added and heated in thermal cycler using programme shown in Table 2.4.

Table 2.4. PCR conditions for Patatin gene

PCR programme	**Temperature**	**Time.**
Initial denaturation	98^0C	2 min
Denaturation	95^0C	30 Sec
Annealing	55^0C	30 Sec
Extension.	72^0C	40 Sec
Final extension	72^0C	5 min
Number of cycles: **35**		

The specificity of the patatin primer set was tested by running a PCR assay with tomato, maize and potato DNA.
Samples that gave positive results (expected fragment) were used for GMO screening PCR assays.

2.8.2. GMO Screening PCR Systems

In order to detect the presence of foreign genetic elements in samples, GMO screening was done using three different PCR assays. The 35S- Promoter, Nos terminator and nptII genes were the targets at the screening stage.

2.8.2.1. Screening for 35S promoter

Primer set 35S-F/35S-R was used to detect 35S promoter in DNA samples. The concentrations of reaction components are shown in Table 2.5.

Table 2.5. Concentrations of PCR components for 35S-Promoter

PCR components	Final concentrations
Sterile ddH20	20.8 µl
10X PCR buffer	3.0 µl (1X)
25 mM $MgCl_2$	3.0 µl (2.5mM)
10 mM dNTP mix	0.6 µl (0.2mM)
35S-F	0.4 µl (0.5µM)
35S-R	0.4 µl (0.5µM)
Taq DNA polymerase	0.2 µl

For 35S promoter, 2 µl of DNA was added to reaction mixture and the thermal cycling programme used for 35S promoter is shown in Table 2.6.

Table 2.6. PCR conditions for 35S- Promoter

PCR programme	Temperature	Time
Initial deneturation	95^0C	2 min
Denaturation	95^0C	30 Sec
Annealing	54^0C	1 min
Extension	72^0C	1 min
Final extension	72^0C	5 min
Number of cycles: 30		

2.8.2.2. NptII specific PCR

Detection of nptII gene was done using primer sets Kn-F and Kn- R. The concentrations of reaction components are shown in Table 2.7

Table 2.7**.** Concentrations PCR components for nptII gene

.

PCR components	Final concentrations
Sterile ddH20	19.6 µl
10X PCR buffer	3.0 µl (1X)
25 mM $MgCl_2$	1.8 µl (1.5mM)
10 mM dNTP mix	0.6 µl (0.2mM)
Kn-F	0.4 µl (0.5µM)
Kn-R	0.4 µl (0.5µM)
Taq DNA polymerase	0.2 µl

For nptII PCR, 4 µl of DNA was added and thermal cycling conditions are shown in Table 2.8.

Table 2.8. PCR conditions for amplification of nptII gene.

PCR programme	Temperature	Time
Initial denaturation	95^0C	3 Min
Denaturation	94^0C	30 Sec
Annealing	53^0C	1 Min
Extension	72^0C	1 Min
Final extension	72^0C	5 Min
Number of cycles: 35		

2.8.2.3. Nos3 terminator specific PCR

Primer set Nos-F/Nos-R was used for detection and amplification of nos3 terminator gene. The concentrations of reaction components are shown in Table 2.9.

Table 2.9. Concentrations of PCR components for Nos terminator

PCR components	Final concentrations
Sterile ddH20	18.6 µl
10X PCR buffer	3.0 µl (1X)
25 mM $MgCl_2$	4.8 µl (4.0mM)
10 mM dNTP mix	0.6 µl (0.2mM)
Nos-F	0.4 µl (0.5µM)
Nos-R	0.4 µl (0.5µM)
Taq DNA polymerase	0.2 µl

After adding reaction mix in individual tubes, 2 µl of DNA was added and thermal cycling conditions used for the amplification of Nos3 terminator are shown in Table 2.10.

Table 2.10. PCR conditions for amplification of Nos terminator gene

PCR conditions	Temperature	Time
Initial denaturation	95^0C	3 min
Denaturation	95^0C	30 sec
Annealing	55^0C	1 min
Extension	72^0C	1 min
Final extension	72^0C	10 min
Number of cycles: 30		

2.8.3. Synthetic Cry 3A Specific PCR

After GMO screening for 35S promoter, nptII and Nos3 terminator genes, the samples where any of these genes were detected were used to conduct synthetic cry3A specific PCR. As of 2003, all approved transgenic potatoes were transformed with synthetic Cry 3A gene for resistance against Colorado potato beetle (BATS, 2003). The concentrations of PCR components for synthetic Cry 3A are shown in Table 2.11.

Table 2.11. Concentrations PCR components for synthetic cry3A gene

PCR components	Final concentrations
Sterile ddH20	19.4 µl
10X PCR buffer	3.0 µl (1X)
25 mM $MgCl_2$	3.0 µl (4.0mM)
10 mM dNTP mix	0.6 µl (0.2mM)
3A-F	0.4 µl (0.5µM)
3A-R	0.4 µl (0.5µM)
Taq DNA polymerase	0.2 µl

After preparation of reaction mixture, 3 µl of DNA was added and the PCR conditions used are shown in Table 2.12

Table 2.12. PCR conditions for amplification of synthetic cry3A gene.

PCR programme	Temperature	Time
Initial deneturation	98^0C	2 Min
Denaturation	94^0C	30 Sec
Annealing	52^0C	1 Min
Extension	72^0C	1 Min
Final extension	72^0C	10 Min
Number of cycles: 35		

2.8.4. Amplification of Btt DNA

For the amplification of Btt DNA, the concentrations of reaction components are shown in Table 2.13.

Table 2.13. Concentrations of PCR components for Btt DNA

Reaction component	Final concentration
Sterile ddH_20	21 µl
10X PCR Buffer	3 µl (1X)
25 mM $MgCl_2$	2.4 µl (2.0mM)
10 mM dNTP mix	0.6 µl (0.2mM)
NC3A-F	0.4 µl (0.5µM)
NC3A-R	0.4 µl (0.5µM)
Taq DNA polymerase	0.2 µl

To the reaction mixture, 2 µl of Btt DNA was added and thermal cycling conditions used was; initial denaturation 95^0C, 4 min; denaturation 95^0C, 30 sec; annealing

51^0C, 1 min; extension 72^0C, 1 min; final extension 72^0C, 5 min. For the amplification of Btt DNA with synthetic primers 3A-F and 3A-R, the same concentrations and PCR conditions (Table 2.11 and Table 2.12) as for synthetic cry3A were used.

For analysis of PCR products, 1.5 - 2% agarose was used. Agarose gel was prepared as described previously. To determine the size of the PCR products, 100 bp DNA ladder Plus (Fermentas#SM0321) or 200 bp DNA ladder were used.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. DNA Isolation

The extraction of high quality DNA is very important for any molecular analysis (Surzycki, 2000). Potato tubers contain large amounts of carbohydrates, a reason why DNA extraction from potato is usually performed with fresh leaves (Wulff et al., 2002). The action of molecular enzymes is inhibited when the extracted DNA contains polysaccharides.

Using a CTAB method with slight modifications, a high molecular weight and high quality DNA was obtained from most samples as seen on agarose gel (Fig 3.1). Modifications made in CTAB method were skipping DNA pellet washing using Buffer 1 and Buffer 2 of Acetate salts. With such modification, still our isolated DNA was workable with Taq DNA polymerase.

Fig 3.1. Genomic DNA agarose gel

Table 3.1. Lanes for genomic DNA agarose gel.

Figure 3.1			
Lanes	Sample	Lanes	Samples
Lane 1	G1	Lane 16	H2
Lane 2	X1	Lane 17	Z2
Lane 3	R	Lane 18	S
Lane 4	K1	Lane 19	U1
Lane 5	N1	Lane 20	Z1
Lane 6	C3	Lane 21	P1
Lane 7	D	Lane 22	S1
Lane 8	M3	Lane 23	14
Lane 9	Y1	Lane 24	T1
Lane 10	F1	Lane 25	T2
Lane 11	A2	Lane 26	B
Lane 12	M2	Lane 27	4
Lane 13	L	Lane 28	13
Lane 14	H1	Lane 29	Tz
Lane 15	R1	Lane 30	B1

The quality of DNA was further verified by measuring its UV absorbance (A) at 260 nm and 280 nm. The ratio A260/A280 provides an estimate of the purity of nucleic acids. The ratio values ranged from 1.7-2.2. The recommended A260/A280 value for high pure DNA is 1.8 and a value of more than 2.0 indicates presence of RNA. Whereas the value of less than 1.8 indicates presence of proteins, our sample of value 1.7 was still amplifiable in our PCR systems. The concentrations of DNA samples were estimated by comparing DNA samples with DNA markers of known concentrations and it ranged from 5 to 20 ng/µl.

3.2. Patatin specific PCR

Patatin is major storage protein gene comprising 40% of the total soluble protein of potato tubers (Zsofia et al., 1996). The patatin gene is specific to solanaceae family encoded by a multigene family.

DNA extracted from all samples was subjected to patatin specific PCR to determine if the DNA is amplifiable. This was aimed at preventing false negatives due to

unamplifiable DNA. Primer set Pat-F and Pat-R was used and the expected amplicon size was 124 bp. All extracted DNA tested with patatin PCR assay showed a very intense 124 bp amplicon (Fig 3.2). The intensity of the amplicon could be due to patatin occurring in high multicopy of up to 40% in potato.

The PCR results for amplification of patatin gene are shown in Fig 3.2.

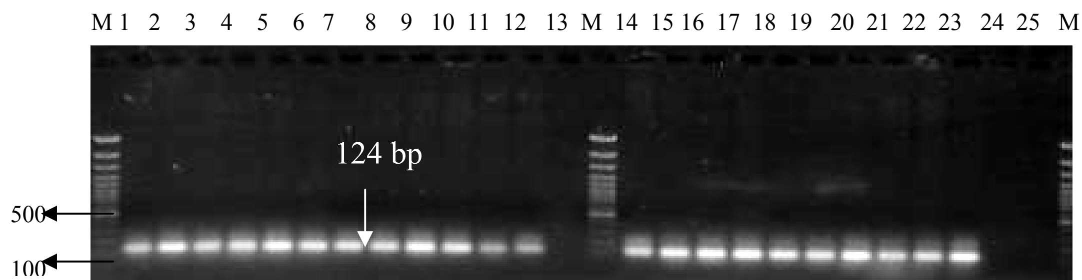


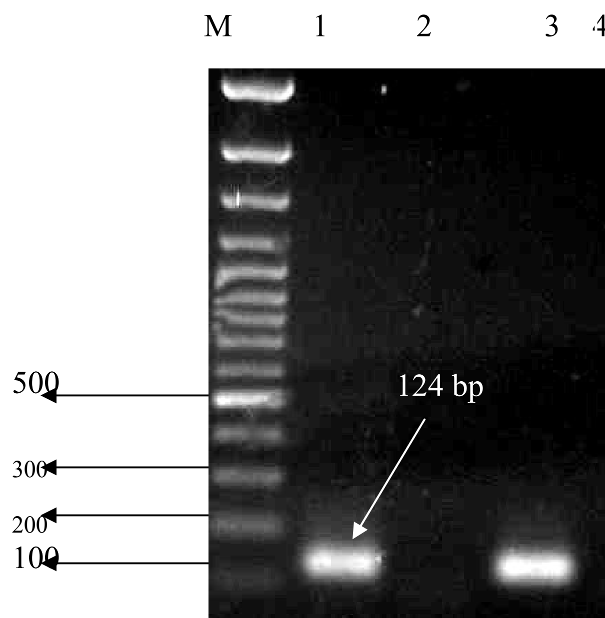
Fig 3.2. PCR results for Patatin gene. Lane M, 100bp ladder (Fermentas#SM0321)

Table 3.2. Lanes for patatin specific PCR products

Figure 3.2			
Lanes	Sample	Lanes	Samples
Lane 1	X1	Lane 16	13
Lane 2	H1	Lane 17	B1
Lane 3	H2	Lane 18	T2
Lane 4	G1	Lane 19	C3
Lane 5	R	Lane 20	K1
Lane 6	T1	Lane 21	S
Lane 7	M2	Lane 22	U1
Lane 8	M3	Lane 23	L
Lane 9	Z1	Lane 24	Empty
Lane 10	Z2	Lane 25	Empty
Lane 11	P1		
Lane 12	N1		
Lane 13	Reagent control		
Lane 14	R1		
Lane 15	14		

Expected bands of Patatin in the 8 additional samples (Y1, F1, D, Tz, A2, S1 and B) were also detected (data not shown).

The specificity of patatin primer set was determined by running a PCR assay using DNA extracted from Maize, tomato and potato. Primer set Pat-F/Pat-R amplified and generated the expected 124bp fragment in potato and tomato DNA, and not with maize DNA (Fig 3.3). Tomato and potato both belong to the Solanaceae family and patatin gene is only present in this plant family thus the observed fragment. Due to lack of patatin gene in maize, the fragment (124 bp) was not observed in maize DNA. This clearly showed that the primer set Pat-F/Pat-R were specific to solanaceae family to which potato belongs.



,

Fig 3.3. The specificity of patatin primer set Pat-F and Pat-R. Lane M, 100 bp DNA ladder; lane 1, Potato DNA; lane 2, Maize DNA; lane 3, tomato DNA; lane 4, reagent control.

3.3. NptII specific PCR

The nptII gene is derived from the prokaryotic transposon 5. The expression of this gene confers resistance to the antibiotic kanamycin and it’s used as a selection marker in developing transgenic plants. Inserting this gene allows to select plant cells that contain the transgene.

All the transgenic potato lines so far approved according to BATS report as of 2003 have nptII gene. Potato DNA samples were tested for the presence of nptII gene using primer set Kn-F/Kn-R. The expected 459 bp fragment was detected in 10 samples of the total 30 (Fig 3.4).

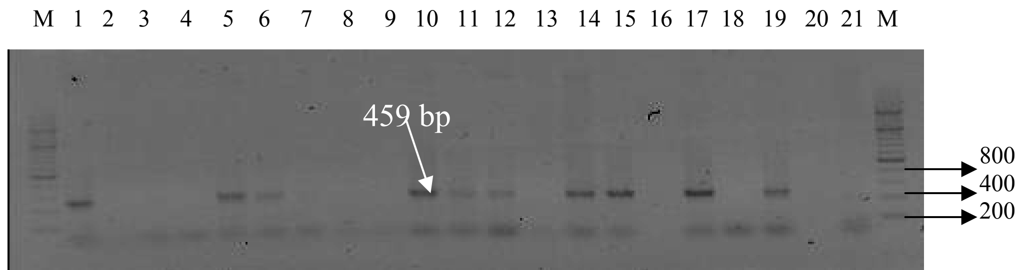


Fig. 3.4. PCR results for nptII gene. Lane M, 200 bp DNA ladder.

Table 3.3. Lanes for nptII PCR products

Fig 3.4 Lanes	Sample	Lane	Sample
Lane1	14	Lane 12	Z2
Lane 2	H1	Lane 13	B
Lane 3	M2	Lane 14	D
Lane 4	Y1	Lane 15	H2
Lane 5	G1	Lane 16	Tz
Lane 6	M3	Lane 17	Positive control
Lane 7	R1	Lane 18	Reagent control
Lane 8	A2	Lane 19	C3
Lane 9	13	Lane 20	Empty
Lane 10	K1	Lane 21	
Lane 11	R		

In sample 14, lane 1; sample G1, lane 5; sample M3, lane 6; sample K1, lane 10; sample R, lane 11; sample Z2, lane 12; sample D, lane 14; sample H2, lane 15 and sample C3, lane 19, the expected band was detected.

The expected band of nptII in 10 additional samples (T1, X1, Z1, P1, S1, N1, B1, T2, U1, and S1) was not detected; however in sample F1, a 459 bp expected fragment was also detected (data not shown).

3.4. P-35S specific PCR

The use of the promoter is to regulate the transcription of the transgene and the choice of the promoter is very important so as to achieve the desired expression levels (Primrose et al., 2001). A constitutive 35S promoter from CaMV was used in many GM crops (MacCormick et al., 1998). Of the total approved GM crops, 56% contain 35S-Promoter (BATS, 2003). However there are many mutants of 35S promoter and their sequences are not identical (Anklam et al., 2002).

When developing Monsanto's Newleaf Atlantic and Russet Burbank lines, 35S-Promoter was used (BATS, 2003). Unlike Atlantic lines, the Russet Burbank lines have both normal 35S promoter (P-35S) and an enhanced 35S promoter (P-E35S). The P-E35S contains a duplicated nucleotide sequence at positions 1116-1368 of the 35S promoter (Jaccaud et al., 2003). Therefore using Primer set 35S-F/35S-R generates two fragments from P-E35S; the 195 bp and 457 bp (Pietsch et al., 1997), whereas only 195 bp fragment is obtained from P-35S. In 5 Samples the expected fragment of 195 bp was detected, indicating they could be Newleaf lines since P-35S was used only for Newleaf lines.

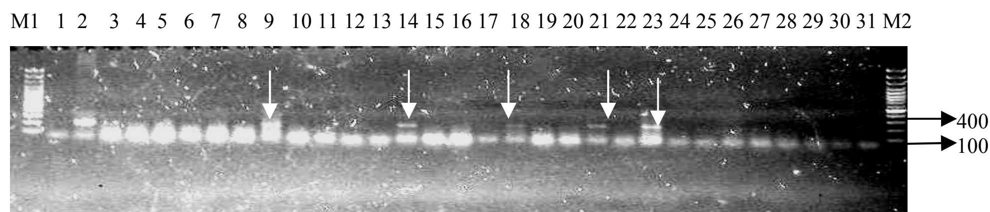


Fig 3.5. PCR results for amplification of 35S promoter. Lane M1, PstI digested lambda DNA ladder; lane M2, 100 bp ladder. White arrows indicate expected 195 bp fragments.

Table 3.4. Lanes for 35S promoter PCR products

Fig 3.5			
Lane	Sample	Lane	Sample
1	Reagent control	16	Y1
2	Positive control	17	4
3	Tz	18	K1
4	U1	19	Z1
5	A2	20	T1
6	S	21	G1
7	13	22	P1
8	D	23	R
9	C3	24	R1
10	Z2	25	F1
11	H2	26	M3
12	14	27	L
13	S1	28	B
14	X1	29	M2
15	H1	30	T2
		31	N1

In samples C3, lane 19; sample X1, lane 14; sample K1, lane 18; sample R, lane 23 and sample G1, lane 21, the expected 195 bp fragment of 35S-Promoter was detected. Sample B1 was giving smears without expected band (data not shown).

Since the positive control (Russet Burbank line) had both P-35S and P-E35S, we expected to detect both 195 bp and 457 bp. However it was only a 195 bp fragment that was obtained. This could be due to PCR conditions that favor annealing of primers to the template DNA position for generating a 195 bp and not the 457 bp fragment.

3.5. Nos3-T specific PCR

The most frequently used terminator in approved transgenic crops is the T-Nos3, for terminating the transgene transcription (BATS, 2003). It has been used in at least

16 of the 28 GMO products (57%), and in all approved transgenic potato lines, T-Nos was used (BATS, 2003).

The Nos3 terminator was isolated from the nopaline synthase gene of *Agrobacterium tumefaciens* (MacCormick et al., 1998).

Primer set Nos-F/Nos-R was used to target and amplify a 180 bp fragment. The expected fragment was detected in 5 of 30 samples. PCR results are shown in Fig 3.6.

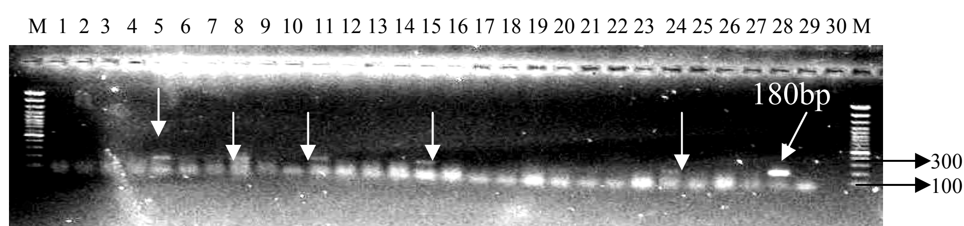


Fig 3.6. PCR results for amplification of T-Nos3. Lane M, 100 bp DNA ladder. White arrows indicate the expected 180 bp fragments.

Table 3.5. Lanes for T-Nos PCR products

Fig 3.6.			
Lane	Sample	Lane	Sample
1	R1	16	B1
2	H1	17	S
3	P1	18	T2
4	H2	19	G1
5	R	20	Y1
6	K1	21	L
7	B	22	U1
8	C3	23	A2
9	N1	24	F1
10	X1	25	T1
11	14	26	Z2
12	Z1	27	Tz
13	D	28	Positive control
14	S1	29	Reagent control
15	M3	30	Empty

In sample R, lane 5; sample C3, lane 8; sample 14, lane 11; sample M3, lane 15 and sample F1, lane 24; the expected 180 bp fragment was obtained. In Samples 4, M2 and 13, T-Nos3 was not detected and data is not shown.

The intensities of Nos terminator PCR products were however faint, and even for the positive control; the T-Nos3 fragment was not as intense as for other genes. This could be attributed to low sensitivity of Nos terminator conventional PCR assay. The T-Nos PCR assay was less sensitive compared to other PCR systems. DMSO was added to increase the fragment intensities however it had no effect.

DNA samples where at least one of the regulatory genes (35S-P and Nos-T), and nptII was detected; were used for conducting synthetic cry3A specific PCR.

3.6. Synthetic Cry3A specific PCR

Native *cry* genes do not function optimally in plants and are therefore not adequate for control of less susceptible insects in the field. To have high expression levels in plants, several modifications were done on the *cry3A* gene. The Synthetic cry3A gene conferring resistance to the Colorado potato beetle was genetically engineered into potato plant (Perlak et al., 1993).
Primer set 3A-F/3A-R was used to target and amplify synthetic cry3A in DNA samples; and expected 479 bp fragment was obtained in 9 of 30 DNA samples (Fig 3.7).

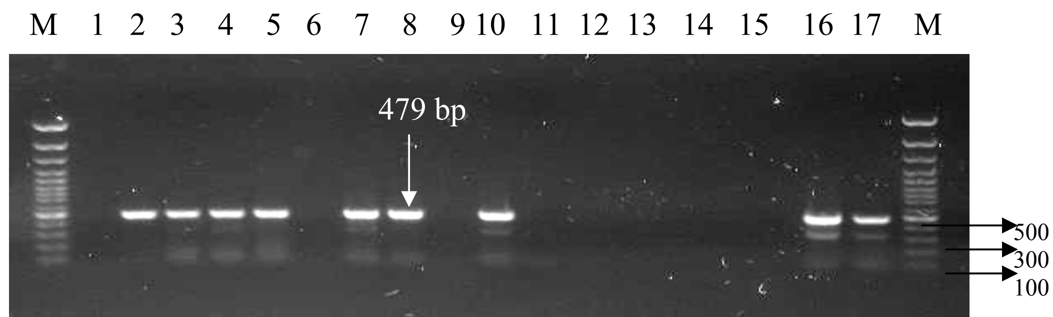


Fig 3.7. PCR results for synthetic cry3A gene. Lane M, 100 bp DNA ladder;

Table 3.6. Lanes for synthetic cry3A specific PCR products

Fig 3.7			
Lanes	Sample	Lanes	Sample
Lane 1	Empty	Lane 8	H2
Lane 2	R	Lane 9	X1
Lane 3	14	Lane 10	Positive control
Lane 4	C3	Lane 11	Reagent control
Lane 5	D	Lane 12-15	Empty
Lane 6	K1	Lane 16	M3
Lane 7	G1	Lane 17	Z2

In sample R, lane 2; sample 14, lane 3; sample C3, lane 4; sample D, lane 5; sample G1, lane 7; sample H2, lane 8; sample M3, lane 16 and sample Z2, lane 17, the expected synthetic cry3A gene was detected. Also in sample F1, the expected fragment was detected but data is not shown.

From the GMO screening and synthetic cry3A specific PCR assays, some samples showed matching results, while on contrary others not.

In all samples where synthetic cry3A gene was detected, nptII gene was also detected (14, C3, M3, G1, H2, Z2, R, F1 and D). This was expected since in all transgenic potato lines so far approved according to BATS report as of 2003, synthetic cry3A, Nos3-terminator and nptII genes were all incorporated. Thus it was expected to detect T-Nos3 as well wherever synthetic cry3A and nptII were detected. However of these samples, T-Nos3 was detected in only samples C3, R, M3, 14 and F1. Failure to detect T-Nos3 in other samples (G1, H2, Z2 and D) could be attributed to the low sensitivity of T-Nos3 conventional PCR assay. As reported above, T-Nos conventional PCR fragment intensities were very low. Our previous study results however have shown that RT-PCR can detect the T-Nos3 where the conventional PCR failed (Gamze, 2004). The samples were T-Nos3 was not detected yet it was expected can be analyzed for T-Nos with RT-PCR.

In sample C3, M3, F1, 14 and R, T-Nos3, nptII and synthetic cry3A gene were detected. It was in only sample R and C3 where all transgenes i.e. nptII, Nos3-T, 35S-P and synthetic cry3A were detected.

In Samples G1 and K1, both 35S-P and nptII gene were detected at GMO screening stage, whereas the synthetic cry3A gene was detected only in sample G1. In sample X1, only 35S promoter was detected. In samples Z2, H2, and D, only nptII gene and synthetic cry3A genes were detected.

Of the 9 samples where synthetic cry3A and nptII was detected, T-Nos was detected in only 5 of these samples, and 35S-P in 3 of these samples.

The 35S-P was not used in all the approved transgenic potato lines, yet Nos-T, nptII and synthetic cry3A genes were all incorporated (BATS, 2003). Therefore Nos3-T, nptII and synthetic cry3A gene could be detected in samples (14, F1 and M3) and 35S-Promoter not. Also in samples H2, Z2, and D where nptII and synthetic cry3A genes were detected and 35S-P not.

The samples (14, F1, M3, H2, Z2 and D) where 35S-P was not detected yet synthetic cry3A, nptII and/or Nos3-T were detected could be lines having novel viral resistance. This is because transgenic potato lines resistant to PLRV or PVY had no 35S-Promoter gene incorporated (BATS, 2003). Detection for viral resistance was not aimed in this study.

In samples T1, N1, A2, M2, L, H1, R1, B, U1, B1, Tz, Y1, S, 4, S1, Z1, P1, T2 and 13, neither of the regulatory gene (35S-P and Nos-T), nptII nor synthetic cry3A gene was detected.
Of the total 30 samples, 11 samples had at least one of the genetic elements detected.

3.7. Specificity of PCR systems

The specificity of all primer sets used in the study was tested in PCR assays using template DNA extracted from transgenic and non-transgenic potatoes. The expected PCR products from 35S-P, T-Nos, nptII and synthetic cry3A genes were amplified from transgenic potato, whereas no amplification products were obtained from non-transgenic potatoes (Fig 3.8).

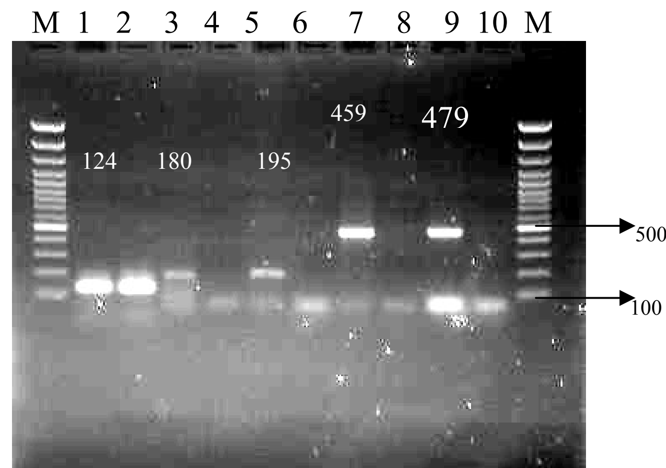


Fig 3.8. Specificity of PCR assays for transgenic vs. non-transgenic potatoes. Lane M, 100 bp DNA ladder; lane 1 and 2, Patatin specific PCR; lane 3 & 4, Nos-T specific PCR; lanes 5 & 6, 35S-P specific PCR; lanes 7 & 8, nptII specific PCR; lanes 9 & 10, synthetic cry3A specific PCR. Lanes 1, 3, 5, 7 & 9, transgenic potato; lanes 2, 4, 6, 8 & 10, non-transgenic potato.

The specificity results clearly indicated that the PCR assays were only specific for genetic elements transformed in transgenic potato lines. Amplification of patatin gene in both transgenic and non transgenic potato DNA (lanes 1 & 2) showed that there was no inhibition of PCR assays.

3.8. Confirmation of PCR products

The most reliable way to confirm the authenticity of a PCR product is its sequencing (DMIF-GEN, 1999). For confirmation of synthetic cry3A PCR products, the fragment of sample R and sample H2 were sent for sequencing. The sequences of cry3A amplicons were compared to synthetic cry3A sequences and several natural cry3A sequences found in Genbanks (Fig 3.9 and Fig 3.10). This was aimed at checking if PCR fragments generated could be that of naturally occurring cry3A gene found in soil bacterium, *Bacillus thuringiensis tenebrionis*, which could in turn give false positive results.

```
Y00420   CGTAGTAGGTGATCTCCTAGGCGTAGTAGGTTTCCCGTTTGGTGGAGCGCTTGTTTCG-T 836
M30503   CGTAGTAGGTGATCTCCTAGGCGTAGTAGGTTTCCCGTTTGGTGGAGCGCTTGTTTCG-T 471
M37207   CGTAGTAGGTGATCTCCTAGGCGTAGTAGGTTTCCCGTTTGGTGGAGCGCTTGTTTCG-T 839
AJ237900 CGTAGTAGGTGATCTCCTAGGCGTAGTAGGTTTCCCGTTTGGTGGAGCGCTTGTTTCG-T 839
U10985   CGTAGTAGGTGATCTCCTAGGCGTAGTAGGTTTCCCGTTTGGTGGAGCGCTTGTTTCG-T 839
PCR^H2   -------------------GTGGAGGTTGGATGCCGNTCNGG--GGGCCCTCGCTGAGGN 39
X70979‡  CGTTGTGGGAGACCTCTTGGGCGTGGTTGGATTTCCCTTCGGTGGAGCCCTCG-TGAGCT 130
                            *  *  ** ** *  *  *  **  * ** ** * *  *

Y00420   TTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTATGG 896
M30503   TTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTATGG 531
M37207   TTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTATGG 899
AJ237900 TTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTATGG 899
U10985   TTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTATGG 899
PCR^H2   NCTATACAAACTTNCTCAACGCGNTTTGGCCAAGCGAGGACCCTTGGAAAGCATTCATGG 99
X70979‡  TCTATACAAACTTTCTCAACACCATTTGGCCAAGCGAGGACCCTTGGAAAGCATTCATGG 190
         ***********  * **  *  ********** ** ***** ***** ** ** ****

Y00420   AACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTCTTG 956
M30503   AACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTCTTG 591
M37207   AACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTCTTG 959
AJ237900 AACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTCTTG 959
U10985   AACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTCTTG 959
PCR^H2   AGCAAGTTGAAGCTCTTATGGATCAGAAGATTGCAGATTATGCCAAGAACAAGGCTTTGG 159
X70979‡  AGCAAGTTGAAGCTCTTATGGATCAGAAGATTGCAGATTATGCCAAGAACAAGGCTTTGG 250
         * ***** *****  * *********** ** ** ******** ** ** ** *** * *

Y00420   CAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 1016
M30503   CAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 651
M37207   CAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 1019
AJ237900 CAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 1019
U10985   CAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 1019
PCR^H2   CAGAACTCCAGGGCCTTCAGAACAATGTGGAGGACTACGTGAGTGCATTGTCCAGCTGGC 219
X70979‡  CAGAACTCCAGGGCCTTCAGAACAATGTGGAGGACTACGTGAGTGCATTGTCCAGCTGGC 310
         ****  * *********** ** ***** ** ** ** ************      ****
```

Figure 3.9. (continued).

```
Y00420   AAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGTTTT 1076
M30503   AAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGTTTT 711
M37207   AAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGTTTT 1079
AJ237900 AAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGTTTT 1079
U10985   AAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGTTTT 1079
PCRH2    AGAAGAACCCTGTTAGCTCCAGAAATCCTCACAGCCAAGGTAGGATCAGAGAGTTGTTCT 279
X70979‡  AGAAGAACCCTGTTAGCTCCAGAAATCCTCACAGCCAAGGTAGGATCAGAGAGTTGTTCT 370
         * ** ** ***** ** **  ******* ** ***** **  **** ****** **** *

Y00420   CTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACGAGG 1136
M30503   CTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACGAGG 771
M37207   CTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACGAGG 1139
AJ237900 CTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACGAGG 1139
U10985   CTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACGAGG 1139
PCRH2    CTCAAGCCGAATCCCACTTCAGAAATTCCATGCCTAGCTTTGCTATCTCCGGTTACGAGG 339
X70979‡  CTCAAGCCGAATCCCACTTCAGAAATTCCATGCCTAGCTTTGCTATCTCCGGTTACGAGG 430
         ******* ***   ** **  * ***** ******   ***** ** ** ** *******

Y00420   TTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAGACG 1196
M30503   TTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAGACG 831
M37207   TTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAGACG 1199
AJ237900 TTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAGACG 1199
U10985   TTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAGACG 1199
PCRH2    TTCTTTTCCTCACTACCTATGCTCAAGCTGCCAACACCCACTTGTTTCTCCTTAAGGACG 399
X70979‡  TTCTTTTCCTCACTACCTATGCTCAAGCTGCCAACACCCACTTGTTTCTCCTTAAGGACG 490
         **** ** ** ** ** ***** ************** ** ** *** * ** ** ****

Y00420   CTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATAAAA 1256
M30503   CTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATAAAA 891
M37207   CTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATAAAA 1259
AJ237900 CTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATAAAA 1259
U10985   CTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATAAAA 1259
PCRH2    CTCAAATCTATGGAGAAGAGTGGGGATACGAGAAAGAGGACATTGCTGANNNNNNNNNNN 459
X70979‡  CTCAAATCTATGGAGAAGAGTGGGGATACGAGAAAGAGGACATTGCTGAGTTCTACAAGC 550
         ******* *********** *********** ***** ** ********
```

Fig 3.9. Sequence alignment of sample H2 fragment with synthetic and natural cry3A sequences. ‡ Synthetic gene accession number; PCR^{H2}, PCR product sequences for sample H2.

The sequence alignment of cry3A fragment of sample R with synthetic and natural cry3A genes is shown in Figure 3.10 below.

```
Y00420   CGTAGTAGGTGATCTCCTAGGCGTAG---TAGGTTTCCCGTTTGGTGGAGCGCTTGTTTC 834
M30503   CGTAGTAGGTGATCTCCTAGGCGTAG---TAGGTTTCCCGTTTGGTGGAGCGCTTGTTTC 469
AJ237900 CGTAGTAGGTGATCTCCTAGGCGTAG---TAGGTTTCCCGTTTGGTGGAGCGCTTGTTTC 837
M37207   CGTAGTAGGTGATCTCCTAGGCGTAG---TAGGTTTCCCGTTTGGTGGAGCGCTTGTTTC 837
U10985   CGTAGTAGGTGATCTCCTAGGCGTAG---TAGGTTTCCCGTTTGGTGGAGCGCTTGTTTC 837
X70979   CGTTGTGGGAGACCTCTTGGGCGTGG---TTGGATTTCCCTTCGGTGGAGCCCTCG-TGA 127
PCR^R    --------GAGACCTCTTGGGCGTGGAGGTTGGATGCCGNTCNG--GGGCCCTCGCTGA 50
                 * ** *** * ***** *   * ** *  *  *  *  ** ** ** * *

Y00420   G-TTTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTA 893
M30503   G-TTTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTA 528
AJ237900 G-TTTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTA 896
M37207   G-TTTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTA 896
U10985   G-TTTTATACAAACTTTTTAAATACTATTTGGCCAAGTGAAGACCCGTGGAAGGCTTTTA 896
X70979   GCTTCTATACAAACTTTCTCAACACCATTTGGCCAAGCGAGGACCCTTGGAAAGCATTCA 187
PCR^R    GGNNCTATACAAACTTNCTCAACGCGNTTTGGCCAAGCGAGGACCCTTGGAAAGCATTCA 110
         *    ***********  * **  *  ********** ** ***** ***** ** ** *

Y00420   TGGAACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTC 953
M30503   TGGAACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTC 588
AJ237900 TGGAACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTC 956
M37207   TGGAACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTC 956
U10985   TGGAACAAGTAGAAGCATTGATGGATCAGAAAATAGCTGATTATGCAAAAAATAAAGCTC 956
X70979   TGGAGCAAGTTGAAGCTCTTATGGATCAGAAGATTGCAGATTATGCCAAGAACAAGGCTT 247
PCR^R    TGGAGCAAGTTGAAGCTCTTATGGATCAGAAGATTGCAGATTATGCCAAGAACAAGGCTT 170
         **** ***** *****  * *********** ** ** ******** ** ** ** ***

Y00420   TTGCAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCAT 1013
M30503   TTGCAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCAT 648
AJ237900 TTGCAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCAT 1016
M37207   TTGCAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCAT 1016
U10985   TTGCAGAGTTACAGGGCCTTCAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCAT 1016
X70979   TGGCAGAACTCCAGGGCCTTCAGAACAATGTGGAGGACTACGTGAGTGCATTGTCCAGCT 307
PCR^R    TGGCAGAACTCCAGGGCCTTCAGAACAATGTGGAGGACTACGTGAGTGCATTGTCCAGCT 230
         * *****  * *********** ** ***** ** ** ** ************      *

Y00420   GGCAAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGT 1073
M30503   GGCAAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGT 708
AJ237900 GGCAAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGT 1076
M37207   GGCAAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGT 1076
U10985   GGCAAAAAAATCCTGTGAGTTCACGAAATCCACATAGCCAGGGGCGGATAAGAGAGCTGT 1076
X70979   GGCAGAAGAACCCTGTTAGCTCCAGAAATCCTCACAGCCAAGGTAGGATCAGAGAGTTGT 367
PCR^R    GGCAGAAGAACCCTGTTAGCTCCAGAAATCCTCACAGCCAAGGTAGGATCAGAGAGTTGT 290
         **** ** ** ***** ** **  ******* ** ***** **  **** ****** ***

Y00420   TTTCTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACG 1133
M30503   TTTCTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACG 768
AJ237900 TTTCTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACG 1136
M37207   TTTCTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACG 1136
U10985   TTTCTCAAGCAGAAAGTCATTTTCGTAATTCAATGCCTTCGTTTGCAATTTCTGGATACG 1136
X70979   TCTCTCAAGCCGAATCCCACTTCAGAAATTCCATGCCTAGCTTTGCTATCTCCGGTTACG 427
PCR^R    TCTCTCAAGCCGAATCCCACTTCAGAAATTCCATGCCTAGCTTTGCTATCTCCGGTTACG 350
         * ******** ***   ** **  * ***** ******   ***** ** ** ** ****

Y00420   AGGTTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAG 1193
M30503   AGGTTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAG 828
AJ237900 AGGTTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAG 1196
M37207   AGGTTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAG 1196
U10985   AGGTTCTATTTCTAACAACATATGCACAAGCTGCCAACACACATTTATTTTTACTAAAAG 1196
X70979   AGGTTCTTTTCCTCACTACCTATGCTCAAGCTGCCAACACCCACTTGTTTCTCCTTAAGG 487
PCR^R    AGGTTCTTTTCCTCACTACCTATGCTCAAGCTGCCAACACCCACTTGTTTCTCCTTAAGG 410
         ******* ** ** ** ** ***** ************** ** ** *** * ** ** *
```

Figure 3.10. (continued)

```
Y00420   ACGCTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATA 1253
M30503   ACGCTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATA 888
AJ237900 ACGCTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATA 1256
M37207   ACGCTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATA 1256
U10985   ACGCTCAAATTTATGGAGAAGAATGGGGATACGAAAAAGAAGATATTGCTGAATTTTATA 1256
X70979   ACGCTCAAATCTATGGAGAAGAGTGGGGATACGAGAAAGAGGACATTGCTGAGTTCTACA 547
PCR^R    ACGCTCAAATCTATGGAGAAGAGTGGGGATACGAGAAAGAGGACATTGCTGANNNNNNNN 470
         ********** *********** *********** ***** ** ********
```

Fig 3.10. Sequence alignment of sample R fragment with synthetic and natural cry3A sequences. ‡ Synthetic gene accession number; PCR^R, PCR product sequences for sample R.

The sequence alignment was done by ClustalW software programme (www.ebi.ac.uk/clustalw, 2/11/2004) and Blast software programme. The homology of cry3A PCR fragments was 93% and 95% with the synthetic cry3A sequences for sample H2 and R respectively. And the score with natural cry3A sequences found in Genbanks was less at 73% (Table 3.7). This rule out the possibility that the PCR fragment using primer set 3A-F/3A-R could have been the natural cry3A gene of Btt. Using Blast software programme, the fragments sequences were identified as synthetic cry3A sequences.

Table 3.7. Comparison of the sequences of fragments generated with synthetic cry3A specific primers with known natural cry3A sequences.

Genbank accession no.	3A-F + 3A-R (479 bp) Homologous region.	Homology (%)
X70979	60-538	93 and 95
Y00420*	764-1213	73
M30503*	401-878	73
U10985*	767-1246	73
AJ237900*	707-1186	73
M37207*	767-1246	73

* Natural cry3A gene accession numbers.

The homology of the synthetic cry3A fragments with natural cry3A genes of 73% is comparable to that reported by Donna et al. (2004) of 76%.

The sequences of the five naturally occurring cry3A sequences were all identical within this region.

Also restriction digestion of PCR products has been reported as alternative method to confirm the authenticity of PCR products (Jaccuad et al., 2003). Analyzing the sequences of both the synthetic and natural cry3A gene showed that, the synthetic cry3A has a PvuII restriction site within the primers 3A-F and 3A-R target region unlike the natural cry3A gene within the region homologous to the amplicon (Fig 3.11).

```
M30503    CAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 651
AJ237900  CAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 959
Y00420    CAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 1016
M37207    CAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 1019
U10985    CAAAATAATGTCGAAGATTATGTGAGTGCATTGAGTTCATGGC 1019
PCR1      AGAACAATGTGGAGGACTACGTGAGTGCATTGTCCAGCTGGC 249
PCR2      AGAACAATGTGGAGGACTACGTGAGTGCATTGTCCAGCTGGC 249
*X70979   AGAACAATGTGGAGGACTACGTGAGTGCATTGTCCAGCTGGC 310
```

Fig 3.11. Sequence alignment of synthetic & natural cry3A genes showing PvuII restriction site (**CAGCTG**); * Synthetic cry3A gene sequences; PCR[1] and PCR[2], PCR product sequences for sample H2 and R respectively.

The expected synthetic cry3A PCR fragment has a PvuII recognition site that when digested two bands 246 bp and 233 bp will be generated. Since the 246 bp and 233 bp have only 13 bp difference, on agarose gel, such a small nucleotides difference cannot be seen, thus restriction of a 479 bp synthetic cry3A PCR products should give an intense band between 200 and 300 bp size ladders (Fig 3.12).

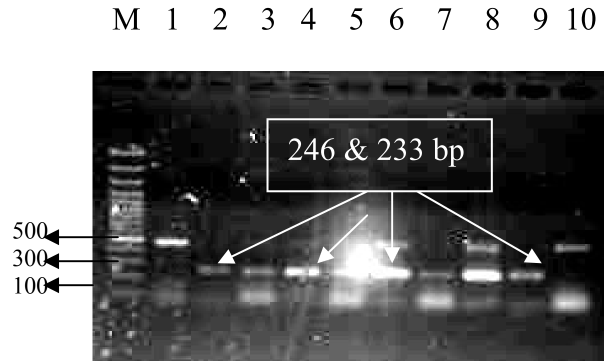


Fig 3.12. PvuII digestion of synthetic cry3A fragments. Lane M, 100 bp DNA ladder; lane 1, undigested positive control; lane 2, positive control; lane 3, sample R; lane 4, sample D; lane 5, sample H2; lane 6, sample F1; lane 7,sample M3; lane 8, sample C3; lane 9, sample Z2; lane 10, undigested sample R.

Restriction digestion of sample 14 and G1 is not shown. Looking at Fig 3.11, samples F1 and C3 were partially digested and the same samples however had the strongest intensities. This could be due to low restriction enzyme amount used in these samples compared to PCR product substrate.

Since all the documented natural cry3A sequences lack this site within the region homologous to the amplicon (Fig 3.11), the cry3A PCR fragments were generated from synthetic sequences because the fragments of sample R, C3, D, H2, M3, F1, and Z2 were restriction digested by PvuII. The cry3A PCR assay therefore did not amplify natural cry3A gene of Btt and there were no false positives in the study.

The possibility that 3A-F and 3A-R primers might have detected the natural cry3A was tested further using Btt DNA (Fig 3.13).

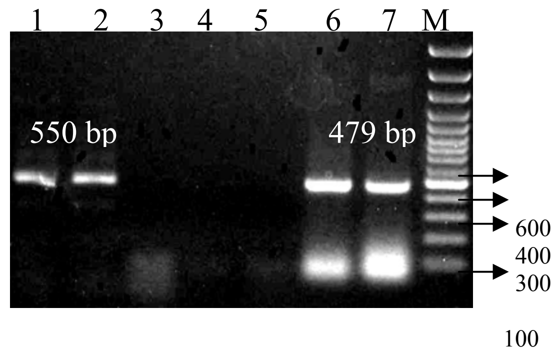


Fig 3.13. Specificity of primers 3A-F and 3A-R for synthetic cry3A: lanes 1 and 2, Btt DNA amplified using natural cry3A primers NC3A-F and NC3A-R; lane 3, reagent control; lanes 4 and 5, Btt DNA using synthetic primers 3A-F and 3A-R; lane 6 and 7, PCR of sample R and H2 respectively using synthetic cry3A primers; lane M, 100 bp ladder.

In Lanes 1 and 2 shows that primers NC3A-F and NC3A-R successfully amplified a 550 bp fragment of natural cry3A gene in Btt DNA. The same Btt DNA containing natural cry3A was analyzed in PCR with primer set 3A-F and 3A-R. There were no amplification products detected using Btt DNA (lanes 4 and 5), whereas samples R and H2 DNA gave the expected fragments (lane 6 and 7) with primers 3A-F and 3A-R. This confirmed that primers 3A-F and 3A-R were specific for synthetic cry3A and that natural cry3A in Btt which could be in the soil associated with potato tubers would not generate false positive results.

3.9. Sensitivity of synthetic Cry3A PCR system

The sensitivity of PCR method for the detection of synthetic cry3A gene in transgenic potato was evaluated using the absolute limit of detection (LOD). Pure DNA from transgenic potato was serially diluted until cry3A gene could no longer be detected. Synthetic cry3A gene could be detected up to 0.001-dilution level, which was equivalent to 10 pg DNA of tuber DNA (Fig 3.14).

This value is comparable to that reported by Jae et al, (2004) of 10 pg and that of Bt maize reported by Jankiewicz et al, (1999).

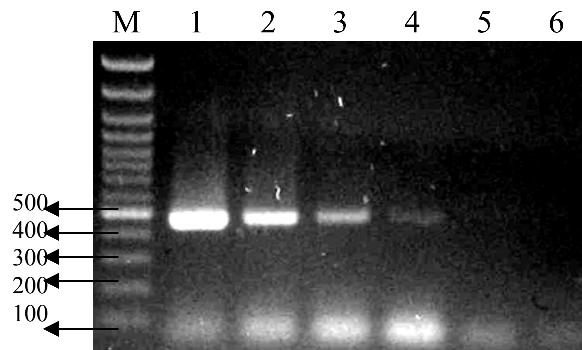


Fig .3.14. Absolute limit of detection in PCR of synthetic cry3A. Lane M, 100 bp ladder; lane 1-5, PCR products generated with 10, 1,0.1, 0.01, and 0.001 ng of DNA respectively; lane 6, reagent control.

The haploid copy of potato genome is estimated to be 0.9 pg (Michael et al., 1999). Thus absolute limit of detection of 10 pg indicated that up to 2.7 genome copies could be detected using this method. The sensitivity of as little as 10pg DNA would be acceptable to ensure verification of non-GMO materials and to monitor the reliability of labeling system (Jae et al., 2004).

3.10. Interpretation of results

The first PCR assay using primers to amplify the patatin gene allowed detecting if the extracted potato DNA was amplifiable. All potato DNA samples gave the expected intense 124 bp fragment, indicating that our DNA was amplifiable and could work with PCR assays.

The specificity of patatin primer set was evaluated using DNA from other plants and it clearly showed that these primers were only specific to potato whose genome contain patatin gene. This was reflected by failure to obtain the expected fragment in maize DNA. Plant specific PCR is important especially if you are to work with materials containing more than one plant species.

Screening methods for detection of transgenic elements in potato samples were established. The T-Nos, P-35S, and nptII genes transformed in most of the approved GM crops were detected in some samples. In addition, the synthetic cry3A transgene transformed in GM potato lines was also detected. Summary of all PCR results obtained are shown in Table 3.8.

Table 3.8. Summary of all PCR results

Sample code	Market place	Patatin	nptII	T-Nos3	P-35S	Cry3A
G1	Ankara	+	+	-	+	+
R1	Ankara	+	-	-	-	-
H1	Ankara	+	-	-	-	-
L	Belgium	+	-	-	-	-
M2	Ankara	+	-	-	-	-
A2	Ankara	+	-	-	-	-
F1	Afyon	+	+	+	-	+
Y1	Ankara	+	-	-	-	-
M3	Ankara	+	+	+	-	+
D	Ankara	+	+	-	-	+
C3	USA	+	+	+	+	+
N1	Ankara	+	-	-	-	-
K1	Eskişehir	+	+	-	+	-
R	Ankara	+	+	+	+	+
X1	Ankara	+	-	-	+	-
H2	Ankara	+	+	-	-	+
Z2	İzmir	+	+	-	-	+
S	Ankara	+	-	-	-	-
U1	Istanbul	+	-	-	-	-
Z1	Izmir	+	-	-	-	-
P1	Ankara	+	-	-	-	-
S1	Ankara	+	-	-	-	-
14	Ankara	+	+	+	-	+
T1	Ankara	+	-	-	-	-
T2	Istanbul	+	-	-	-	-
B	Ankara	+	-	-	-	-
4	Ankara	+	-	-	-	-
13	Ankara	+	-	-	-	-
Tz	Tanzania	+	-	-	-	-
B1	Ankara	+	-	-	-	-
15 (Transgenic)	USA	+	+	+	+	+
Q (Non transgenic)	Niğde	+	-	-	-	-

+, detected; **-,** not detected.

In sample R and C3 from Ankara and USA respectively T-Nos, P-35S, nptII and synthetic cry3A genes were all detected. Since all these genes were transformed in transgenic Newleaf insect resistant potato lines, samples R and C3 were transgenic.

In Sample G1; 35S promoter, nptII and synthetic cry3A genes were detected. As outlined above, the T-Nos PCR assay was not very sensitive in some samples and failure to detect T-Nos in sample G1 does not rule it out to be transgenic, since screening PCR systems are only indicative for the presence of transgene, in this case synthetic cry3A (Anklam et al., 2002).

Detection of P-35S specifies the Newleaf insect resistant potato lines only. Samples G1, R and C3 where P-35S were detected in addition to other genetic elements were therefore transgenic Newleaf insect resistant lines. The 35S promoter was transformed in only insect resistant Newleaf potato lines (BATS, 2003). However, there could be other GM potatoes other than Newleaf lines whose genetic elements have not been disclosed.

In sample M3, 14 and F1, T-Nos, nptII and synthetic cry3A genes were detected. The 35S promoter was not detected in these samples. As mentioned above the 35S promoter was not transformed in transgenic potato lines with virus resistance, yet nptII, T-Nos and synthetic cry3A gene were all transformed. Failure to detect 35S promoter is samples M3, 14 and F1, therefore categorizes them into transgenic lines with both viral and insect resistance. These potato lines could not be characterized for the different viral resistances (PLRV and/or PVY) because it was not aimed in this study.

In samples D, Z2, and H2, only nptII and synthetic cry3A genes were detected. We expected to detect T-Nos as well in these samples since nptII and Nos3 terminator genes were transformed together with the synthetic cry3A gene when developing GM potato lines. As reported earlier above, Nos3 terminator PCR assay was not very sensitive in some samples compared to other PCR assays.

By both sequencing and restriction digestion, all the cry3A PCR products were confirmed to be synthetic sequences, further more primers 3A-F and 3A-R were found to amplify only synthetic sequences of cry3A. Samples D, 14 and H2 were therefore transgenic even though T-Nos was not detected in these samples.

Of the total 30 market samples, synthetic cry3A gene was detected in 9 samples (C3, R, M3, Z2, F, D, H2, G1 and 14). Failure to detect T-Nos in some of these 9 samples does not render them non-transgenic since nptII and T-Nos and 35S-Promoter are only indicative for the presence of synthetic cry3A or any other transgene.
This follows therefore that 9 of the 30 samples were found to have synthetic cry3A transgene transformed in transgenic potato lines to resist CPB. The samples were synthetic cry3A was detected were therefore transgenic lines. The cry3A gene detected in these samples was not the natural cry3A gene found in soil bacterium (*Bacillus thuringiensis tenebrionis*) from sequencing results, restriction digestion and testing cry3A primer set with natural cry3A gene.

The discrepancy in the detection of T-Nos with synthetic cry3A and nptII genes in some samples could be attributed to PCR sensitivity. Real Time PCR can probably work better for T-Nos3 detection, since it's more sensitive compared to conventional PCR.

In sample K1, only 35S promoter and nptII were detected, and in sample X1, it was only 35S promoter detected. Besides insect and viral resistant transgenic potato lines developed by Monsanto, there are some transgenic potatoes sighted in literature. For example, changin potato, developed by Switzerland Company had nptII gene incorporated and in the same potato T-Nos3 was detected (Anklam et al., 2002). Also detection of 35S promoter has been reported in one potato line, whose specification was not known (Anklam et al., 2002). In B33 invertase potato

developed by IGF Germany, GM desire potato and GM Rustica potato engineered with gbss antisense constructs, nptII gene was detected (Anklam et al., 2002).

Also Spunta-G2 and Spunta-G3 that are resistant to potato tuber moth were developed by Michigan State University in conjunction with Egypt. These transgenic potatoes were released for commercialization (http:/www.msu.edu/douchesd/commercialRelease, 11/12/2004). Such transgenic potato lines and many more whose T-DNA constructs used for transformation are not published, could be on market and samples K1 and X1 could fall in such categories.

In samples L and Tz from Belgium and Tanzania respectively T-Nos, P-35S, nptII and synthetic cry3A genes were not detected, yet their DNA was amplifiable according to PCR results of patatin gene. These samples were therefore non-transgenic.

Samples T1, M2, B, H1, P1, N1, Z1, 4, S1, A2, T2, Y1, U1, S, R1, B1 and 13, neither T-Nos3, P-35S, nptII nor synthetic cry3A gene was detected. Unless when there are other genetic elements that were transformed in some transgenic potato lines rather than P-35S, T-Nos, nptII, and synthetic cry3A that were investigated in this study, these samples were non-transgenic.

In summary, 35S promoter gene was detected in 5 samples; Nos terminator in 5 samples; nptII gene in 10 samples and synthetic cry3A in 9 samples. NptII gene was detected wherever synthetic cry3A was detected. Failure to detect T-Nos in some samples that had nptII and synthetic cry3A detected could be attributed to less sensitivity of T-Nos conventional PCR assay.

CHAPTER 4

CONCLUSIONS

In Turkey, potato (patates) is ranked 4th in order of importance to other crops (CIP, 1998). There are consumed in a number of forms and quite a number of fast food products processed from potatoes are available on the market.

Available information shows that Turkey's trade partners in potato seeds include Romania, USA, Israel and Canada, and in all these partner countries, GM potatoes were approved for cultivation. As of 2003, published information shows that the synthetic Cry3A gene that confers resistance to Colorado potato beetle was transformed in all approved transgenic potato lines (BATS, 2003).

This study aimed to detect if genetically modified potatoes exist in Turkish food market, using DNA based PCR method.

In 11 samples at least one of the transformed genetic elements was detected. Synthetic cry3A was detected in 9 of total 30 samples. In sample R obtained from Ankara, all genetic elements transformed in transgenic Newleaf insect resistant potato lines were detected. In samples F1, M3 and 14 from Afyon, and Ankara respectively, failure to detect 35S promoter categorizes them in transgenic Newleaf virus and insect resistant potato lines. Detection of synthetic cry3A allowed specific characterization of the transgenic potato lines so far approved.

The conventional PCR system for the detection of T-Nos was however not sensitive enough in some samples. The samples in which the T-Nos3 was not detected yet it

was expected can be analyzed for T-Nos using Real Time-PCR since it's more sensitive than conventional PCR.

Our results have shown the presence of GM potatoes on food market. Thus in the light of those results, it is recommended and emphasized that more potato samples all over Turkey should be collected and examined in an accredited GMO detection laboratory to obtain official results.

On the other hand, the national biosafety GMO legislation regulating GMOs in Turkey has already been prepared and will be implemented soon. The legislation has come at right time when genetically modified foods on food markets are starting to increase.

REFERENCES

Alexander A.T. Johnson1 & Richard E. V **(2003).** Integration of transgenes into sexual polyploidization schemes for potato (*Solanum tuberosum* L.). *Euphytica* **133:** 125–138.

Aluizio B, Fabricio R and Bowen D **(2003).** Understanding biotechnology. Pearson Education Inc. NJ. pp 45-60.

Angus Reid Group Inc**. (1999).** International Awareness and Perceptions of Genetically Modified Foods. The Economist/Angus Reid Poll. Nov/Dec, 1999.

Angus Reid Group Inc. (**2000**). Significant Knowledge Gap in Debate over Modified Foods.

Anklam E, Gadani F, Heinze P, Pijnenburg H, Van den Eede G **(2002).** Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant derived food products. *European Food Research Technology* **214**: 3-26

Anon **(1996).** Agriculture: Humble crop goes high-tech!. *Science News* **150** 5, pp 73b.

Anon **(1998).** Success of edible vaccines may depend on picking the right fruit. The Scientist August 18, 1998: 4–5.

Arce, P., M. Moreno, M. Gutierrez, M. Gebauer, P. Dell'Orto, H. Torres, I. Acuna, P. Oliger, A. Venegas, X. Jordana, J. Kalazich & L. Holuigue **(1999).** Enhanced resistance to bacterial infection by *Erwinia carotovora* subsp. *atroseptica* in transgenic potato plants expressing the attacin or the cecropin SB-37 genes. *Am J Potato Res* **76**: 169–177.

BATS **(2003).** Genetically modified crops. Molecular and Regulatory details. http://www.gmo-watch.org

Barker, H., B. Reavy, K.D. McGeachy & S. Dawson (1999). A unique form of transgenic resistance to potato mop-top virus induced by transformation with the coat protein gene. *Phyton* **39**: 47–50.

Bonfini L., Heinze P., Kay S., Van den Eede G **(2001).** Review of GMO detection and quantification techniques**.** European Commission, Joint Research Centre. Institute for Health and Consumer Protection, Final report.

Braun R **(1999).** The Public Perception of Biotechnology in Europe: Between Acceptance and Hysteria. Biolink, Communication on Biotechnology, August 1999.

Byrdwell WC, Neff WE **(1996).** *J Liq Chromatogr Relat Technol* **19**:2203–2225.

CBAC **(2002).** Improving the Regulation of Genetically Modified Foods and Other Novel Foods in Canada. Canadian Biotechnology Advisory Committee, Ottawa.http://cbac-cccb.ca/

Cellini F., Chesson, A., Colquhoun, I., Constable, A., Davies, H.V., Engel, K.H., Gatehouse, A.M.R., Kaerenlaempi, S., Kok, E.J., Leguay, J.J., Lehesranta, S., Noteborn, H.P.J.M., Pedersen J., Smith M **(2004).** Unintended effects and their detection in genetically modified crops. *Food and chem. Toxicology* **42**:1089-1125.

CFIA **(1998).** Import Permit Requirements for Plants with Novel Traits (Including Transgenic Plants), and their Products, Directive D-96-13 of July 27, 1998. Plant Health and Production Division, Plant Products Directorate, Canadian Food Inspection Agency, Nepean.

Clive J (**2003**). Preview: Global Status of Commercialized Transgenic Crops: 2003. *ISAAA Briefs* No. 30. ISAAA: Ithaca, NY.

ClustalW software programme www.ebi.ac.uk/clustalw **(2/Nov/2004)**

Cockburn A **(2002).** Assuring the safety of genetically modified (GM) foods: the importance of a holistic, integrative approach. *Journal of Biotechnology* **98:** 79–106.

Codex Alimentarius Commission **(2003).** Draft Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants. Joint FAO/WHO Food Standards Programme, Food and Agriculture Organisation, Rome.

Conway G **(1999).** The doubly green revolution: food for all in the twenty-first century. Cornell University Press.

Conway G., and G. Toenniessen **(1999).** Feeding the world in the twenty-first century. *Nature* 402(6761) Suppl: C55-58.

Crist W.E **(1996).** Waiter, there's a flounder in my fruit. (Bio-engineered fruits and vegetables with animal genetic materials are not so labeled). *Vegetarian Times* **231:** 22.

Daie J, Belanger F **(1993).** Plant factories: production of industrial proteins and non-food products in transgenic plants. *Agro Food Industry Hi-Tech* **January-February**, 1993: 7.

De Lumen B.O., Krenz, D.C. and Jamela, M **(1997).** Molecular strategies to improve the protein quality of legumes. *Food Technol* **51** 5, pp. 67–70.

Dickson D **(1999).** Proceed with caution, says UK report on ethics of GM foods. *Nature* **399** 6735: 396.

DMIF-GEN **(1999).** Development of methods to identify foods produced by means of genetic engineering. EU-Project SMT4- CT96–2072. DMIF-GEN Final Report, 15 December 1999

Donna S. S, Philip W. M, Solke H. D **(2004).** Method for the detection of synthetic cry3A in transgenic potatoes. *J. Agric. Food Chem*. **52**:809-815.

European Commission **(2002).** Safety approval for placing on market of potato line EH92-527-1. Directorate-general xxiv, Scientific Committee on Plants.

European Commission (**2003).** Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Official Journal of the European Communities L* **268**, pp. 1–23.

European Commission **(1997).** Regulation (EC) No 258/97 of the European Parliament and of the Council concerning novel foods and novel food ingredients. *Official Journal of the European Communities L*43, 1–7.

European Commission **(2001).** Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Official Journal of the European Communities* **L106**, pp. 1–39.

European Commission **(2002a).** Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities* **L31**, pp. 1–24.

European Commission **(2003).** Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. *Official Journal of the European Communities L* **268**, pp. 24–28.

FAO/WHO **(2000).** Safety Aspects of Genetically Modified Foods of Plant Origin. Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, Geneva, Switzerland, 29 May–2 June 2000. http://www.fao.org/es/esn/food/gmreport.pdf

FAO/WHO **(1991).** Strategies for Assessing the Safety of Foods Produced by Biotechnology, Report of a Joint FAO/WHO Consultation. , World Health Organisation, Geneva.

FAO/UNICEF **(1998).** The state of the world's children 1998. New York: Oxford. University Press for UNICEF.

Ferré F (**1998**). Key issues, challenges, and future opportunities in gene quantification. In: Ferré F (ed) Gene quantification. Advanced biomedical technologies. Birkhäuser, Boston, pp 1–16.

Food Marketing Institute (FMI) (**1995**). Trends in the United States: Consumer Attitudes and the Supermarket, Food Marketing Institute, Washington, DC.

FSANZ **(2000).** Standard 1.5.2, Food Produced using Gene Technology. Food Standards Australia New Zealand, Canberra and Wellington. http://www.sciencedirect.com/science

FSANZ **(2001).** Information for Applications, Amending Standard A18/Standard 1.5.2 -Food Produced using Gene Technology. Food Standards Australia New Zealand, Canberra and Wellington. http://www.sciencedirect.com/science

Gadani F, Bindler G, Pijnenburg H, Rossi L, Zuber J **(2000)** Beitraege zur Tabakforschung.

Gamze A (2004). Detection of genetically modified maize via polymerase chain reaction (unpublished).

Goto, F., T. Yoshihara, N. Shigemoto, S. Toki, and F. Takaiwa **(1999).** Iron fortification of rice seed by the soybean ferritin gene. *Nature Biotechnology* 17**(3):** 282-86.

Gray S.N **(1998).** Fungi as potential bioremediation agents in soil contaminated with heavy or radioactive metals. *Biochem Soc Trans* 26**(4)**: 666–670.

Hallman W.K. and Metcalfe J **(1994)**. Public perceptions of agricultural biotechnology: a survey of New Jersey residents. Rutgers University, Piscataway, NJ.

Hassairi, A., K. Masmoudi, J. Albouy, C. Robaglia, M. Jullien & R. Ellouz **(1998**). Transformation of two potato cultivars 'Spunta' and 'Claustar' (*Solanum tuberosum*) with lettuce mosaic virus coat protein gene and heterologous immunity to potato virus Y.

Hauman B.F **(1997).** Bio-engineered oilseed acreage escalating. *Inform* **8:** 804–811.

Hefferon, K.L., H. Khalilian & M.G. AbouHaidar **(1997).** Expression of the PVY(O) coat protein (CP) under the control of the PVX CP gene leader sequence: protection under greenhouse and field conditions against PVY(O) and PVY(N) infection in three potato cultivars. *Theor Appl Genet* **94**: 287–292.

Hemmer W **(1997)** Foods derived from genetically modified organisms and detection methods. BATS-Report 2/1997, Agency for Biosafety Research and Assessment of Technology Impacts of the Swiss Priority Programme Biotechnology of the Swiss National Science Foundation, Basel, Switzerland.

Hileman B **(1999)**. Prescription for a global biotechnology dialogue. *Chemical & Eng News* 77(**29**): 42.

Hoban T.J **(1999).** Consumer acceptance of biotechnology in the United States and Japan. *Food Technology* **53(**5): 50–53.

Hoban, T.J. May (**1996).** Consumers Will Accept Biotech Foods. BT Catalyst.

Hupfer C, Hotzel H, Sachse K, Engel KH **(1997)** Z Lebensm Unters Forsch 205: 442–445.

Hurburgh CR, Rippke GR, Heithoff C, Roussel SA, Hardy CL **(2000).** Detection of genetically modified grains by Near infrared spectroscopy. Proceedings PITTCON 2000 – Science for the 21st Century, #1431. New Orleans, La. 12–17 March 2000.

Ibrahim SM, Lofts RS, Jahrling PB, Hencal EA, Weedn VW, Northrup MA, Belgrader P **(1998)**. *Analytical Chemistry* **70**:2013– 2017.

International Food Information Council (IFTC) (**2000**). U.S. Consumer Attitudes Toward Food Biotechnology. (Wirthlin Group Quorum Survey).

International potato center (ICP) http://www.cippotato.org/market/potatofacts **(2000).**

ISAAA (International service for acquistion of agri-biotech applications) http://www.isaaa.org (**2003**).

Jaccaud E, Höhne M, Rolf M **(2003).** Assessment of screening methods for the identification of genetically modified potatoes in raw materials and finished products. *J. Agric. Food Chem.***51**: 550-557.

Jackson W **(1991).** Development of perennial grains. Paper presented at the 18th International conference on the Unity of the Sciences. Aug 23–26, 1991, Seoul Korea.

Jae K R, Theresa L, Soon-IL J and Tae-San K (**2004**). Qualitative and quantitative PCR methods for detection of three lines of genetically modified potatoes. *J. Agric. Food Chem.* **52**: 3269-3274.

Jankiewicz A., Broll H., Zagon J **(1999).** The official method for the detection of genetically modified soybeans and insect resistant Bt maize (Maximizer). *Eur. Food res. Technol.* 209: 77-82.

Japan MAFF **(1995).** Guidelines for Application of Recombinant DNA Organisms in Agriculture, Forestry, Fisheries, the Food Industry and Other Related Industries. Innovative Technology Division, Research Council Secretariat, Ministry of Agriculture, Forestry and Fisheries, Tokyo. http://www.sciencedirect.com/science

Japan MHLW **(2000).** Procedures of Application for Safety Assessment of Foods and Food Additives Produced by Recombinant DNA Techniques. Ministry of Health, Labour, and Welfare, Tokyo. http://www.sciencedirect.com/science

Jasanoff S **(2000).** Commentary: between risk and precaution—reassessing the future of GM crops. *Journal of Risk Research* **3**: 277–282.

Jennifer A. T **(2000).** The genetically modified foods debate in South Africa. UCT Monday Paper, February 2000.

Kaiser J **(1999).** Animal rights: activists ransack Minnesota labs. *Science* **284:** 410–411

Kaiser J **(1996).** Pests overwhelm Bt cotton crop. *Science* **273(**5274): 423.

Kitamura K **(1995).** Genetic improvement of nutritional and food processing quality in soybean. *Japan Agric Research Quarterly* **29:** 1–8.

Koch K **(1998).** Food safety battle: organic vs. biotech. *Congressional Quarterly Researcher* **9** (33): 761–784.

König A **(2003).** A framework for designing transgenic crops—science, safety, and citizen's concerns. *Nature Biotechnology* **21:**1274–1279.

König, Cockburn, Crevel, Debruyne, Grafstroem, Hammerfing, Kimber, Knudsen, Küiper, Penninks, poulsen, Schauzu, Wal **(2004).** Assessment of the safety of foods derived from GM crops. *Food and Chemical toxicology*, **42**:(7) 1047-1088.

Köppel E, Stadler M, Lüthy J, Hübner P **(1997)** Mitt Geb Lebensm Hyg **88**: 164–175.

Laane C, and Willis B.J (**1993**). Biotechnology in food production. In: Encyclopedia of Food Sciences: *Food Technology and Nutrition,* vol. 1. Acad Press, New York, 392–95.

Leech, M. J., K. May, D. Hallard, R. Verpoorte, V. De Luca, and P. Christou **(1998).** Expression of two consecutive genes of a secondary metabolic pathway in transgenic tobacco: molecular diversity influences levels of expression and product accumulation. *Plant Molecular Biology* 38**(5):** 765-74.

Lesney M.S **(1999).** The Garden of Dr. Moreau: plant biotechnology as a chemical enterprise. Genetically modified foods are the fore-runners of designer chemical plants. *Todays Chemist at Work* 8(**6):** 28–33.

Li, W., K.A. Zarka, D.S. Douches, J.J. Coombs, W.L. Pett & E.J. Grafius **(1999).** Coexpression of potato PVY. coat protein and cryV-Bt genes in potato. *J Am Soc Hort Sci* **124**: 218–223.

Lin H. Y, Chiueh L.C, Shih Y.C (2000). Detection of genetically modified soybeans and maize by the polymerase chain reaction. *Journal of Drug and Food Analysis* **8**:200-207.

Liu K.S. and Brown E.A **(1996).** Enhancing vegetable oil quality through plant breeding and genetic engineering. *Food Technol* **50(**11): 67–71.

MacCormick, C.A., Griffin, H.G., Underwood, H.M., and Mason, M.J (**1998**). Common DNA sequences with potential for detection of genetically manipulated organisms in food. *J. Appl. Microbiol.* **84**: 969-980.

Maki-Valkama, T., T. Pehu, A. Santala, J.P.T. Valkonen, K. Koivu, K. Lehto & E. Pehu **(2000).** High level of resistance to potato virus Y by expressing P1 sequence in antisense orientation in transgenic potato. *Mol Breed* **6**: 95–104.

Martina N.M and Burke J.I **(2000).** Biotechnology. Present position and future development. Teagasc, Ireland. pp 51- 80.

Meyer R **(1995)** Z Lebensm Unters Forsch 201: 583–586.

Meyer R Jaccaud E **(1997)** Detection of genetically modified soy in processed food products: Authenticity and adulteration of food – *The analytical approach,* vol 1. Interlaken, Switzerland 24–26 September **1997:** 23–28.

Michael D and Ilia J **(1997).** Nuclear DNA amounts in angiosperms- 583 new estimates. *Annals of botany* **80**:169-197.

Mohammed, A., D.S. Douches, W. Pett, E. Grafius, J. Coombs, L.W. Liswidowati & M.A. Madkour, **(2000).** Evaluation of potato tuber moth (Lepidoptera: Gelechiidae) resistance in tubers of Bt-cry5 transgenic potato lines. *J Econ Entomol* **93**: 472–476.

NAS (National Academy of sciences). Transgenic plants and world Agriculture report, **2000**. http://www.nap.edu/books/NI000027.

National Science Foundation (NSF) (**2000).** Science and Engineering Indicators. (Jack Cooper, Food Industry Environmental Network, June 29, 2000)

Nordlee J.A., Taylor, S.L., Townsend, J.A., Thomas, L.A. and Bush, R.K **(1996).** Identification of Brazil nut allergen in transgenic soybeans. *N Engl J Med* **334:** 668–692.

Nottingham S **(2003).** Eat your gene: How genetically food is entering our diet. 2nd Edition. Zed books limited. pp 156-170.

OECD **(1993).** Safety Evaluation of Foods Derived by Modern Biotechnology, Concepts and Principles. , Organisation for Economic Cooperation and Development, Paris http://www.oecd.org

Palucha, A., W. Zagorski, M. Chrzanowska & D. Hulanicka., **(1998).** An antisense coat protein gene confers immunity to potato leafroll virus in a genetically engineered potato. *Eur J Plant Pathol* **104**: 287–293.

Pehu, T.M., T.K. Makivalkonen, J.P.T. Valkonen, K.T. Koivu, K.M. Lehto & E.P. Pehu, **(1995).** Potato plants transformed with a potato-virus YP1 gene sequence are resistant to PVY-degrees. *Am Potato J* **72**: 523–532.

Perlak F., Stone T., Muskpf Y., Petersen L., Parker G., McPherson S., Wyman J., Love S., Reed G., Biever D., Fischhoff D **(1993)**. Genetically improved potatoes: protection from damage by Colorado potato beetles. *Plant mol. Biol*. **22**:313-321.

Philips S.C **(1994).** Genetically engineered foods: do they pose health and environmental hazards? *CQ Researcher* 4**(29):** 673–696.

Pietsch K, Waiblinger HU, Brodmann P, Wurz A (**1997)** Dtsch Lebensm Rundsch 93**(2)**: 35–38.

Pinto, Y. M., R. A. Kok, and D. C. Baulcombe. (**1999**). Resistance to rice yellow mottle virus (RYMV) in cultivated African rice varieties containing RYMV transgenes. *Nature Biotechnology* 17**(7):** 702-707

Preston LR, Harker N, Holton T, Morell MK **(1999).** Plant Var Seeds **12**:191–205.

Reed, G.L., A.S. Jensen, J. Riebe, G. Head & J.J. Duan**, (2001).** Transgenic Bt potato and conventional insecticides for Colorado potato beetle management: comparative efficacy and non-target impacts. *Entomol Exptl Appl* **100**: 89–100.

Primer 3 software programme www-genome.wi.mit.edu/cgi-bin/primer/primer3 **(18/06/2004).**

Primrose S.B., Twyman R.M, Old R.W **(2001).** Principles of gene manipulation. 6th edition. Blackwell Science, UK. pp 230.

Rissler J, Mellon M **(1993).** Perils amid the promise: ecological risks of transgenic crops in a global market. Union of Concerned Scientists, Washington D.C., Dec. 1993.

Regulation in Turkey. http://www.tagem.gov.tr **(10/09/2004).**

Rohricht P **(1999).** Transgenic protein production. Part 2. Process economics. *BioPharm* **12:**52–54.

Russell, V., Denny R., Parnett, S., Walton, F., Merriam, D. and Barnes, K **(1987).** The Novo Report: American Attitudes and Beliefs about Genetic Engineering. Research and Forecasts, Inc., New York.

Sanders GWH, Manz A **(2000).** *Trends Anal Chem* **19**: 364–378.

Smaglik. P **(1999).** Food as medicine: nutritionists, clinicians disagree on role of chemo-preventive supplements. *The Scientist* 13(**11**): 14.

Sönmezalp Z.C **(2004).** Detection of genetically modified insect resistant tomato via polymerase chain reaction (unpublished).

Spillane, C., D.C. Baulcombe & T.A. Kavanagh, **(1998).** Genetic engineering of the potato cultivar Glenroe for increased resistance to potato virus X (PVX). *Irish J Agric Food Res* **37**: 173–182.

Spunta potato lines. http://www.msu.edu/douchesd/commercialRelease. (**11/12/2004**).

Surzycki S (2000). Basic techniques in molecular biology. Springer, New York. pp 1-40.

Taylor S. L **(1997).** Food from genetically modified organisms and potential for food allergy. *Environmental Toxicology and Pharmacology* **4**:121-126.

Thayer A.M **(1999).** Transforming agriculture: transgenic crops and the application of discovery technologies are altering the agrochemical and agriculture businesses. *Chemical & Eng News* **77(**16): 21–35.

Threshold value EU. http://www.efsa.eu.int/science/gmo **(15/01/2005).**

Threshold value, Japan. http://www2.ipcku.kansai-u.ac.jp/~kshigeru/res/GMO.pdf, **(15/01/2005).**

Uzogara S. G **(2000**). The impact of genetic modification of human foods in the 21st century. *Biotechnology Advances* 18**(3):179-206.**

Van den Eede G., Aarts, H., Buhk, H.-J., Corthier, G., Flint, H.J., Hammes, W., Jacobsen, B., Midtvedt, T., van der Vossen, J., von Wright, A., Wackernagel, W. and Wilcks, A **(2004).** The relevance of gene transfer to the safety of food and feed derived from GM plants. *Food and Chemical Toxicology* **10**:1016.

Waters LC, Jacobson SC, Kroutchinina N, Khandurina J, Foote RS, Ramsey JM **(1998).** *Analytical Chemistry* **70**:5172–5176.

Windels P, Theuns I, Dendauw J, Depicker A, Van Bockstaele E, M de Loose **(1999).** Development of a line specific GMO detection method: a case study. Meded Fac landbouwwet Rijksuniv. Gent, 64/5b, Gent, Belgium.

Wolf C, Scherzinger M, Wurz A, Pauli U, Hübner P, Lüthy J **(2000)**. *Eur Food Res Technol* **210**:367–372.

Wolters, A.M.A., E.M. Janssen, M.G.M. Rozeboom-Schippers, E. Jacobsen & R.G.F. Visser (**1998).** Composition of endogenous alleles can influence the level of antisense inhibition of granule-bound starch synthase gene expression in tetraploid potato plants. *Mol Breed* **4**: 343–358.

Wulff G., S. Torres and E. Gonzales **(2002).** Protocol for DNA extraction from potato tubers. *Plant molecular biology reporter* **20**:187a-187e.

Wurz A **(1999).** The use of screening procedures and highly specific PCR systems for the analysis of transgenic foods – experiences and implications for routine analyses. In: Zagon J, Broll H, Schreiber GA, Schauzu M (eds) Proceedings of the European Research Project SMT4-CT96–2072: "Development of Methods to Identify Foods Produced by Means of Genetic Engineering". BgVV-Heft 5/1999. Bundesinstitut für gesundheitlichen Verbraucherschutz und eterinärmedizin, Berlin, Germany, pp. 31–36.

Ye, X., S. Al-Babili, A. Kloti, J. Zhang, P. Lucca, P. Beyer, and I. Potrykus. **(2000).** Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287**(5451): 303-5.

Zhen, W., X. Chen, H.B. Liang, Y.L. Hu, Y. Gao & Z.P. Lin **(2000).** Enhanced late blight resistance of transgenic potato expressing glucose oxidase under the control of pathogen-inducible promoter. *Chin Sci Bull* **45**: 1982.

Zsofia B., Attila M and Gergely M **(1996).** Starch synthesis and tuber storage protein genes are differently expressed in solanum tuberosum and in solanum brevidens. *Febs letters* **383**:159-164.

APPENDIX A

CHEMICALS AND SUPPLIERS

CHEMICALS	SUPPLIERS
Ficol 400	Sigma
Agarose	Applichem
NaCl	Merck
NaOH	Merch
CTAB	Applichem
Tris	Sigma
EDTA	Sigma
PVP	Sigma
Hydrogen Peroxide	Sigma
Bromphenol Blue	Sigma
Ethidium Bromide	Sigma
DMSO	Applichem
Glacial Acetic Acid	Applichem
Ethanol	Delta Kimya
Isopropanol	Delta Kimya
Chloroform:Isoamylalcohol	Applichem
HCl	Applichem

APPENDIX B

BUFFERS AND SOLUTIONS

1.1. DNA extraction Buffer (200 ml)

CTAB	4 g
NaCl	16,4 g
Tris HCl	3,15 g
EDTA	1,5 g
Distilled water	100 ml

Add more distilled water to the final volume of 200 ml and adjust pH to 8. Autoclave.

1.2. Washing buffer (70% alcohol)

Distilled water	30 ml
Pure ethanol	70 ml

1.3. 50X TAE Electrophoresis Buffer

2 M Tris base

1 M acetic acid

50 mM Na^2 EDTA

242 g of Tris Base, 57.1ml of acetic acid, 800 ml of double distilled water and 100 ml of 0.5M EDTA are added together and powder dissolved. Water is added to final volume of 1 liter.

1.4. Ethidium bromide stock solution

100 mg ethidium bromide dissolved in 10 ml water.

1.5. Loading dye solution

15% Ficoll 400

40% sucrose

0,01% bromophenol blue

0,01% Xylene cyanol

Dissolve the appropriate amounts in double distilled water

1.6. TE Buffer:

10 ml 1M Tris HCl, pH 8.0

2 ml 0.5M EDTA, pH 8.0

Add distilled water to 1000 ml

Autoclave

APPENDIX C

C.1. Primer binding sites and homology among nptII gene sequences

```
AF274974 GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 540
AF274586 GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 518
AY456412 GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 332
AF274975 GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 404
AY159034 GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 332
         ************************************************************

AF274974 GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 600
AF274586 GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 578
AY456412 GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 392
AF274975 GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 464
AY159034 GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 392
         ************************************************************

AF274974 CCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 660
AF274586 CCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 638
AY456412 CCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 452
AF274975 CCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 524
AY159034 CCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 452
         ***** ******************************************************

AF274974 TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 720
AF274586 TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 698
AY456412 TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 512
AF274975 TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 584
AY159034 TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 512
         ************************************************************

AF274974 CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 780
AF274586 CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 758
AY456412 CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGATGATCTCGTCGTGACCCATGGCGATGC 572
AF274975 CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 644
AY159034 CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 572
         ********************************* **************************

AF274974 CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 840
AF274586 CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 818
AY456412 CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 632
AF274975 CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 704
AY159034 CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 632
         ************************************************************

AF274974 GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 900
AF274586 GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 878
AY456412 GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 692
AF274975 GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 764
AY159034 GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 692
         ************************************************************

AF274974 GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 960
AF274586 GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 938
AY456412 GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 752
AF274975 GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 824
AY159034 GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 752
         ************************************************************
```

Appendix C1 (continued).

```
AF274974                    GCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 1003
AF274586                    GCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 981
AY456412                    GCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 795
AF274975                    GCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 867
AY159034                    GCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 795
```

C.2. Primer binding sites and sequence homology among 35S promoter

```
AY373338 AGGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTC554
A18053   AGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCATTCAAGATGCCTC1141
V00141   AGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTC1244
AF078810 AGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTC564
         ******* ********************************** *** ** **********
AY373338 TGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGA614
A18053   TGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGA1201
V00141   TGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGA1304
AF078810 TGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGA624
         ************************************************************
AY373338 CGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGA674
A18053   CGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGGGA1261
V00141   CGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGA1364
AF078810 CGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGA684
         *************************************** ********************
AY373338 TGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCA734
A18053   TGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCA1321
V00141   TGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCA1424
AF078810 TGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCA744
         ************************************************************
```

C.3. Primer binding sites and sequence homology among Nos terminator sequences

```
AY123624 ATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAA 97
A18053   ATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAA 120
U12540   ATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAA 95
AY562548 -------------------------ATGATTATCATATAATTTCTGTTGAATTACGTTAA 35
                                  ***********************************
AY123624 GCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAG 157
A18053   GCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAG 180
U12540   GCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAG 155
AY562548 GCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAG 95
         ************************************************************
AY123624 AGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGA 217
A18053   AGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGA 240
U12540   AGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGA 215
AY562548 AGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGA 155
         ************************************************************
AY123624 TAAATTATCGCGCGCGGTGTCATCTATGTTACTAGAT----------- 254
A18053   TAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAAGATCC 288
U12540   TAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCG--------- 254
AY562548 TAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATC---------- 193
```