DETECTION AND QUANTIFICATION OF GENETICALLY MODIFIED MAIZE VIA POLYMERASE CHAIN REACTION

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ABSTRACT

DETECTION OF GENETICALLY MODIFIED MAIZE VIA POLYMERASE CHAIN REACTION

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In recent years, foods produced by genetic engineering technology have been on the world food market. The biosafety aspects, regulations, and labelling of these foods are still contentious issues in most countries. It is necessary to have approval for the use of GMOs in the production of food. Thus, detection and quantification of GMOs play crucial role for developing regulations on GM foods.

In this study, raw and processed maize samples were analysed for genetic modification using a DNA based detection method, the Polymerase Chain Reaction. Ten raw food and 18 processed maize food including maize flour, starch, corn flakes, maize chips were collected from different markets located in different places in Turkey. The samples were examined for the presence of genetic elements located in the majority of transgenic crops such as NOS terminator, CaMV 35S promoter, kanamycin resistance (Kan^R) gene, using conventional PCR with oligonucleotide sets targeting to novel genes. Furthermore screening was conducted via Real-Time PCR assay for NOS terminator and 35S promoter. For confirming the presence of Bt11 maize lines event specific primers were utilised. Quantification of Bt11 maize lines were performed via Real-Time PCR.

The result indicates that foreign genetic elements were found in all analysed raw material. In six out of 10 raw material, presence of Bt11 gene were identified. GMO detection was also possible for maize flour and starch, however in processed material as corn starch, corn flakes, corn chips and pop corn, transgenes were not detected.

Keywords: Genetically modified organisms, quantification, PCR, Real time PCR, Genetically modified foods

ÖZ

GENETİK MODİFİYE MISIRLARIN POLİMERAZ ZİNCİR REAKSİYONU İLE SAPTANMASI

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Son yıllarda genetik mühendisliği teknolojisi ile üretilen gıdalar marketlerde görülmektedir. Pek çok ülkede bu gıdaların biyogüvenliği, regülasyonları, etiketlenmeleri hala tartışma konusudur . Pek çok ülkede GMO (genetik modifiye organizma) ların gıda üretiminde kullanılmaları için onaylanmaları gerekmektedir. Bu yüzden GM (genetik modifiye) gıdaların kantitasyon ve tesbit çalışmaları, bu gıdaların yasal düzenlemelerinde önemli rol oynamaktadır.

Bu calışmada, DNA tabanlı tesbit metodu, Polimeraz Zincir Reaksiyonu (PZR), kullanılarak işlenmiş ve işlenmemiş örnekler analiz edildi. Türkiye' nin farklı yerlerinde bulunan farklı marketlerden toplanan GM mısırların analizinde 10 işlenmemiş mısır ürünü ve mısır unu, mısır nişastası, mısır gevreği, mısır cipsi, ve patlamis mısır çesitlerini içeren 18 işlenmiş mısır ürünü incelendi. Örnekler

NOS terminator, CaMV 35S promotor, kanamisin direnç geni gibi transgenik bitkilerin pek çoğunda bulunan genetik elementlerin varlığını göstermek için, yeni genleri hedef alan oligonukleotid setleri ile PZR kullanılarak incelendi. Ayrıca, NOS terminator ve 35S promotor icin eş zamanlı PZR ile tarama yapıldı. Bt11 mısır çesitlerini saptamak için gen kasetine spesifik primerler kullanıldı. Bt11 mısır çesitlerininin kantitasyonu, eş zamanlı PZR ile gerçekleştirildi.

Sonuçlar, analiz edilen işlenmemiş materyallerin hepsinde yabancı genetik elementlerin bulunduğunu gösterdi. 10 işlenmemiş materyalden 6 sında, Bt11 geninin varlıgı saptandı. GMO (genetik modifiye organizma) tesbitinin, mısır unu ve mısır nişastası için mümkün oldugu halde, mısır gevreği, mısır cipsi, ve patlamis mısır gibi işlenmis gıdalarda, yabancı gen tesbiti yapılamadı.

Anahtar kelimeler: Genetik modifiye organizmalar, kantitasyon, polimeraz zincir reaksiyonu, eş zamanlı polimeraz zincir reaksiyonu, Genetik modifiye gıdalar

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ABBREVATIONS

EDTA: Ethlenediamineetetraacetic acid

EtBr: Ethidium Bromide

kb: kilobase

Rpm: Revolution per minute

Uv: Ultra vialote

GMO: Genetically Modified Organisms

GMF: Genetically Modified Foods

GM: Genetically Modified

NOS: Nopaline synthase

CaMV: Cauliflower Mosaic virus

Kan ^{R} : Kanamycin Resistance

PCR: Polymerase Chain Reaction

RT-PCR: Real time Polymerase Chain Reaction

LMO: Living Modified Organisms

EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase

PLRV: potato leaf roll virus

PG: polygalactouronase

ECB: European Corn Borer

Bt: Bacillus thuringiensis

Cry proteins: crystal proteins

ICP: Insecticidal crystal proteins

nptII: neomycin phosphotransferase (type II)

CRM: Certified reference materials

ELISA: Enzyme linked immunosorbent assay

Mb: megabase

EC: European Committee

IRMM: Institue for Reference Materials and Measurements

nm: nanometer

LOD: Limit of detection

Ct: Cycle threshold

Cp: Crossing Point

ND: non detected

e-P-35S: enhanced 35S promoter

CHAPTER I

INTRODUCTION

1.1. Definitions

Genetically Modified Organisms (GMOs) can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating or natural recombination, i.e. by being genetically modified (GM) or by recombinant DNA technology. The addition of foreign genes has often been used in plants to produce novel proteins that confer pest and disease tolerance and, more recently, to improve the chemical profile of the processed product (Anklam et al, 2002, Gachet et al, 1999). GMOs are also named as Living Modified Organisms (LMOs) (Tozzini et al, 2000). Bacteria, fungi, viruses, plants, insects, fish and mammals are examples of organisms that have been artificially changed or altered in order to modify physical properties or capabilities (Gachet et al, 1999).

Genetically modified foods (GMFs) are foods or additives derived from GMOs that are produced or treated via gene modification techniques. GMFs are also named as genetically engineered foods (GEFs) or genetically manipulated foods (Lin et al, 2000).

Plants, animals and micro organisms have been genetically modified and used as food or food additives during last decade (Lin et al, 2000). Most of the GMOs, consumed as food, have been derived from plants (Gachet et al, 1999). Insect resistance, herbicide tolerance, disease resistance, virus resistance plants have been on the world food market.

1.2. Importance of Genetic Modification in Agriculture

Agricultural biotechnology has opened new avenues in the development of plants for the production of food, feed, fibre, forest and other products (Anklam et al, 2001). During the eight year period, 1996 to 2003, the global area of transgenic crops increased 40 fold from 1.7 million hectares in 1996 to 67.7 million hectares in 2003 (James, 2003). Global area of transgenic crops was shown in Figure 1.1

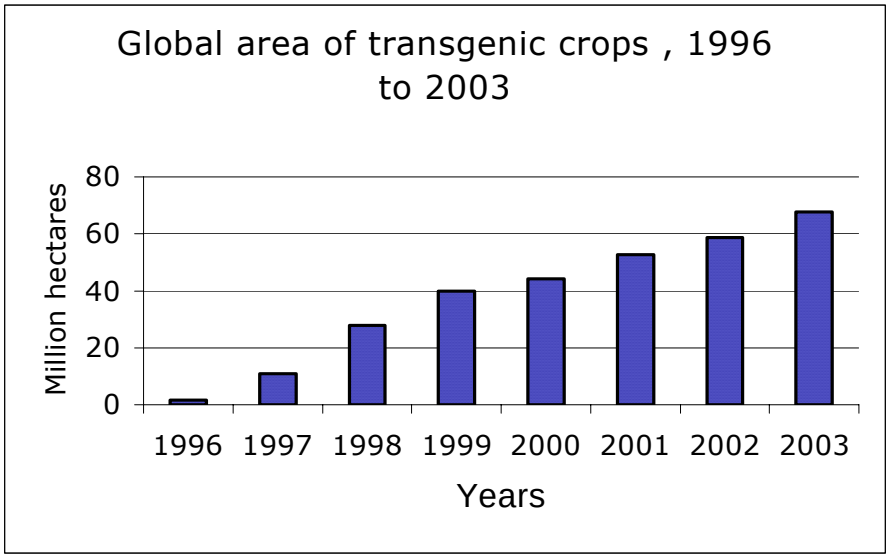


Figure 1.1. Global area of transgenic crops, 1996 to 2003 (James, 2003).

The purpose of developing genetically modified crops is to produce desired properties by altering specific traits of crops. The major traits are herbicide tolerance, insect resistance, virus and disease resistance (Lin et al, 2000).

During the eight year period 1996 to 2003, herbicide tolerance has consistently been the dominant trait with insect resistance being second. Virus resistance and Others as delayed ripening and improved nutrient content are the last.

Distribution of transgenic crops by trait was demonstrated in Figure 1.2 (James, 2003).

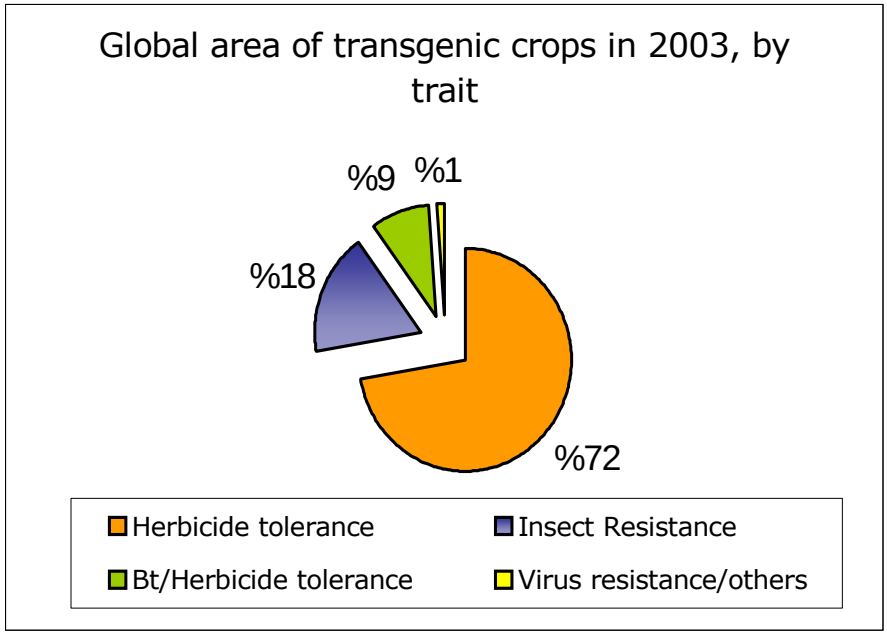


Figure 1. 2. Distribution of transgenic crops by trait (James, 2003).

In recent years global gene modification techniques have been greatly improved. In addition to vector based gene transfer systems, biolistics and micro injection have also been used to create genetically modified crops. The most popular GM-crops for field trials are soybean, tomato, maize, potato, wheat, cotton, sugar beet, oilseed rape, cucumber, melon, lettuce, sunflower, rice and tobacco (Lin et al, 2000).

However only certain types of GMOs have been approved for consumption. The distribution of the global transgenic crop area for the four major crops was illustrated in Figure 1.3.

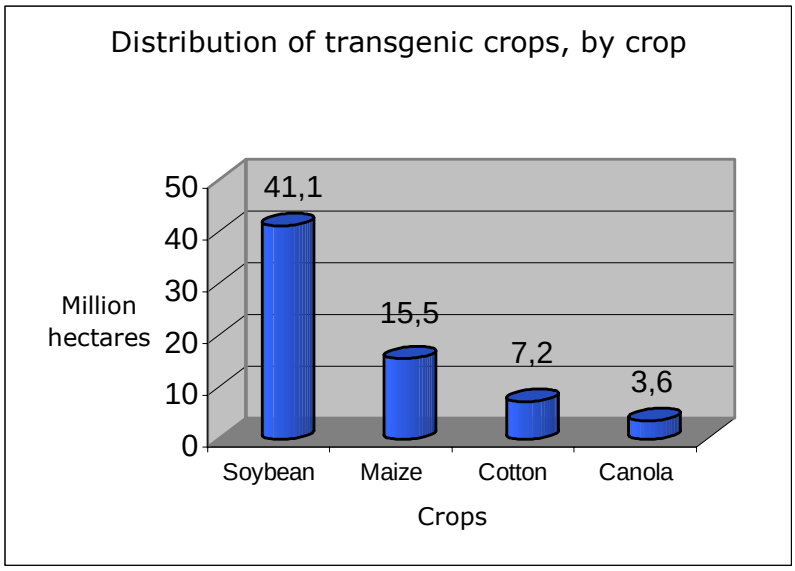


Figure 1.3. Distribution of transgenic crops, by crops in 2003 (James, 2003)

By applying gene modification, potential gains for the global agriculture are the production of higher yields and needing less amount of herbicides and pesticides (Anklam et al, 2001). Loses due to pests and diseases have been estimated at 37% of the agricultural production world-wide, with 14% due to insects (Jouanin et al, 1998, Hilder and Boulter 1999). Pesticide applications may be reduced through the adoptation of Bt containing crops. Reducing pesticide use can improve the health of farmers and consumers, in addition to lowering input costs.

Apart from economical perspective, transgenic methods can be used to improve the micronutrient content and bioavailibility of commonly eaten foods in developing countries (Bouis et al, 2003). Additionally gene modification provides a key role to develop plant with novel traits which have the ability to sustain their presence in the harsh conditions. It results in to obtain new lands with unfavourable growing environments such as those with high saline soils (Hilder and Boulter, 1999).

1.3. Genetically Engineered Novel Foods

Plant biotechnology will likely have the greatest impact on the food industry in the next few years. Some of the traits including enhanced insect-disease-herbicide resistance and improved climatic tolerance are being introduced into many commercially important crops including soybeans, potato, maize, tomato and wheat. Thus the food industry will likely be processing large quantities of genetically modified crops into foods within the coming decade (Taylor, 1997).

First genetically engineered fruit sold on the market was the well-known *FlavrSavr* ® tomato, which was designed to soften more slowly. Currently, the most widely inserted genes in GMOs confer resistance to worms, insects or to a herbicide. One of the aims has been to create a plant that resist any chemical protection used by farmers; for example soybean or corn that are tolerant to herbicides like Round Up. Round Up is a non-selective herbicide which acts by entering the plant and inhibiting an enzyme necessary for building aromatic amino acids. The lack of these aminoacids kills the plant (Gachet et al, 1999). The development of transgenic corn lines with new traits has become one of the main activities of research departments of agro-industry. Insect resistant corns are resistant against the European Corn Borers (ECB), due to the insertion of a sequence coding for a synthetic cryIAb endotoxin (Zimmerman et al, 1998).

A lot of GMOs are under development. For instance, a genetically modified corn altered to make a healthier cooking oil by reducing its saturated fat content is 7on the way. In the case of strawberries, adding an antifreeze protein from the winter flounder fish to help them grow in cold climates is under development, as are nutritionally enhanced strawberries with increased levels of a natural anti-cancer agent, ellagic acid. In the same way, for broccoli, a combination of natural anti-cancer and antioxidant agents could make this cruciferous vegetable essential eating as it would prevent or slow down the aging of cells that form living organisms. Soon, potatoes richer in starch will be used to produce low-fat chips and crisps that one might find in the stores in 5 years time. Banana is also subjected to genetic manipulation studies. Scientists are

investigating whether genetically modified bananas could produce a vaccine against hepatitis B. Rice and lettuce are being engineered. Among GMOs, there are also rape seed resistant to fungi or to herbicides and GM chicory, papaya and squash (Gachet et al, 1999).

Moreover, genetic modification of lactic acid bacteria is achieved either by the inactivation of a gene or by the expression of a gene. Such modifications affects the biochemical pathways resulting in different end products. This in turn affects the texture, yield or quality of the fermented food. Genetically modified yogurts are on the market. In recent years much research has been conducted into the genetic manipulation of yeast in order to enhance indigenous characteristics, such as ethanol tolerance , and to obtain expression of foreign genes and the secretion of foreign proteins which are useful to the food industry (MacCormick et al, 1997).

Fish of many species have been subjected to over a period of more than 10 years but in the last few years have any begun to be considered seriously for the food market. Transgenic fishes have been developing in order to improve cold tolerance /freeze resistant and disease resistant, sterile fish. The production of valuable human pharmaceuticals in GM fish is in progress (Maclean, 2003).

Although seemingly innumerable variety of GM crops has been proposed and developed, only a limited number have been introduced and achieved commercial success (McKeon, 2003).

1.4. Types of Genetic Modifications on Crops

The main commercial and near-commercial applications of crop genetic transformations are as follows.

- **Transformation for insect resistance**

Of the transgenes used, the most common modification involves use of cry genes for protein toxins from the soil bacterium, *Bacillus thuringiensis*. Another

example is transformation of plant derived genes such as those encoding enzyme inhibitors or lectins to develop a source resistance to insects. The major transgenic insect resistance crops are maize, soybean, potatoes, tomatoes, canola, and rice (Jouanin et al., 1998).

- **Transformation for herbicide tolerance**

In plants, the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) plays a key role in the biochemical pathway that results in the synthesis of aromatic amino acids. The major herbicides as glyphosate could selectively inhibits the activity of EPSPS, thus shutting of amino acid synthesis, quickly resulting in plant death. The introduction of a glyphosate-tolerant EPSPS gene, derived from common soil bacterium, *Agrobacterium tumefaciens* strain CP4, forms the basis of glyphosate herbicide tolerance when expressed in GM plants. The major transgenic herbicide tolerance crops are maize, soybean and canola (MacKenzie and McLean, 2002).

- **Transformation for disease resistance**

Transgenic plants containing various parts of the viral genome can be protected against virus. This includes expression of coat protein genes in the target plant. Tomatoes resistant to yellow leaf curl virus , tobacco resistant to tobacco mosaic virus, potatoes resistant to potato leaf roll virus (PLRV), are examples of commercial virus resistant transgenic crops (MacKenzie and McLean 2002).

- **Transformation with desirable quality genes**

Transgenic crops with desirable quality can be important for both food and feed production, although it remains a minor application of genetically engineered crops thus far. The first quality enhanced product on the market was tomatoes containing a lower level of product of the polygalactouronase (PG) gene. The lower activity of this enzyme means that the long pectin chains in the cell walls of the fruit are cleaved more slowly and the ripe fruit does not soften as quickly, thus extending its shelf life (Skerrit, 2000).

- **Transformation for nutritional purposes and pharmaceutical purposes**

Examples of transgenic crops for nutritional purposes include enhanced lysine content in maize and enhanced vitamin A content in canola. However edible vaccines for viral and diarrhoeal diseases using proteins expressed in transgenic plants is an application of potentially huge significance for developing countries. The vaccine is expressed in edible parts of fruit, vegetable, grain plant and has several potential advantages. Multiple vaccines could be produced in one plant (e.g. to all hepatites strains) (Skerrit, 2000).

1.5. Insect Resistant maize

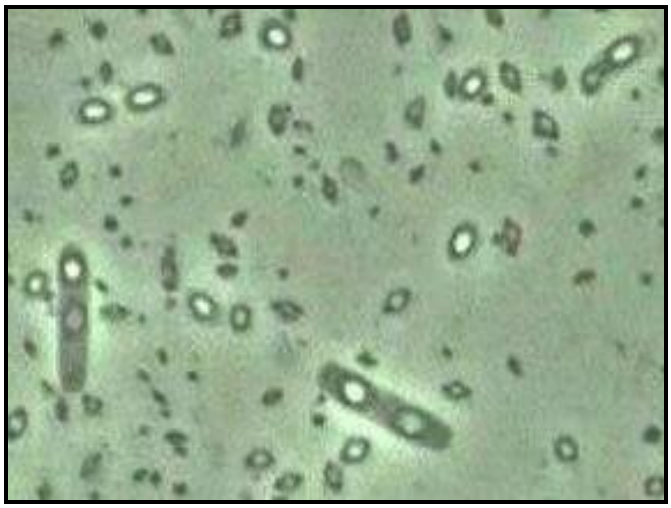
Insect resistant corn was obtained by introducing a bacterial gene *(cry)* coding for an insecticidal protein into the corn genome in order to resist attack by larvae of European Corn Borer (Figure 1.4. and Figure 1.5.). This gene was cloned from a soil bacterium *Bacillus thuringiensis* (Belzile, 2002).

Figure 1.4. Larvae of European corn borer (ECB), (17-08-2004 www.ars.usda.gov/ is/pr/2000/000426.htm)

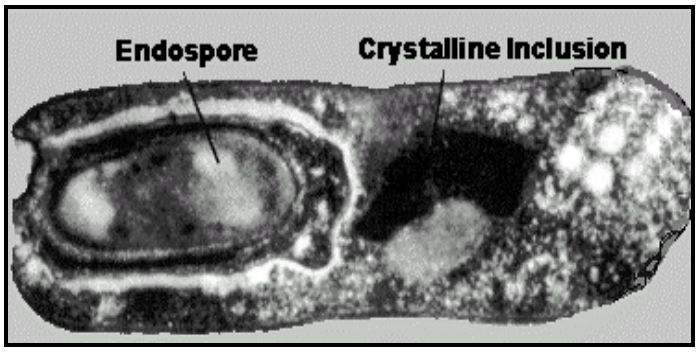
Figure 1.5. European Corn Borer, (17-08-2004 www.inra.fr/.../ HYPPZ/IMAGES/7032440.jpg)

1.5.1. Characteristics of Bt

Bacillus thuringiensis (Bt) is a ubiquitous gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. *B. thuringiensis* was initially characterised as an insect pathogen, and its insecticidal activity was attributed largely or completely to the parasporal crystals (Schnepf, 1995). Microscopic view of Bt was illustrated in Figure 1.6.



(A)



(B)

Figure 1.6. Microscopic view of *Bacillus thuringiensis*,(A) General view, (B) Crystalline inclusions. (www.arches.uga.edu/ ~adang, 21-08-2004)

The insecticidal crystal proteins are commonly designated as "Cry" proteins and the genes encoding them as "cry" genes (De Maagd et al, 1999). These crystalline proteins are highly insecticidal at very low concentrations. This observation led to the development of bioinsecticides based on *B. thuringiensis* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera. Strains have been isolated worldwide from many habitats, including soil, insects, stored-product dust, and deciduous and coniferous leaves (Schnepf, 1995).

1.5.2. History of Bt

Bacillus thuringiensis (Bt) was discovered first in Japan in 1901 in a silkworm rearing unit by Ishawata. In 1911, it was again isolated in a flour moth population and characterised by Berliner in Germany (Jouanin et al, 1998). *Bacillus thuringiensis* formulations were used as insecticidal sprays in the 1930s, but large scale production only started with the introduction of Thuricide™ in the late 1950s and this was followed by similar products from several companies. Cry proteins degrade rapidly in UV light, loosing their

activity. It is therefore necessary to make multiple applications throughout the growing season, which raises the costs of pesticide treatment. Although some improvements have been made in this area, it remains the biggest single drawback to the use of Bt sprays. (De Maagd et al, 1999). Due to photosensitivity, conventional Bt sprays lack persistence and can only protect the plant surface (Brousseau et al, 1999). Thus, Bt sprays are non-systemic insecticides and are therefore ineffective against insects that do not come into direct contact with the crystals, such as sap sucking and piercing insects, against root dwelling pests, or larvae that after hatching rapidly burrow or bore into plant tissues. These problems bring about creating transgenic plants that express the crystal proteins. In these plants, the toxin is continuously produced and protected against degradation, and provided it is expressed in the appropriate tissues it will also be ingested by boring larvae (De Maagd et al, 1999). The first results concerning the transfer of *Bacillus thuringiensis* (Bt) genes in tobacco and tomato were published in 1987. Since then, *Bacillus thuringiensis* (Bt) genes have been transferred to a number of other crop species such as cotton, maize and rice (Jouanin et al, 1998). Different Bt subspecies are effective against different insects as illustrated in Table 1.1.

Table 1.1. Subspecies of Bt types against different insects

Subspecies of the Bt	**Activity**
Bacillus thuringiensis subs *aizawa (Bta)*	Moth larvae of wax moths and other moth species
Bacillus thuringiensis subs *israelensis (Bti)*	Diptera larvae such as mosquitoes, black flies,
Bacillus thuringiensis subs *kurstaki (Btk)*	Lepidopteran larvae such as moths, butterflies, skippers,
Bacillus thuringiensis subs *San Diego*	Larvae of elm leaf beetle
Bacillus thuringiensis subs *tenebrionis (Btt)*	Coleoptera such as potato beettle and the boll weevil

The main approach uses δ-endotoxin coding sequences occurred in 1996 when the first generation of insecticidal plants were introduced on market (Jouanin et al, 1998).

1.5.3. Cry toxins

Cry proteins have been classified according to their insect specificity and nucleotide sequence. Insecticidal crystal proteins (ICPs) were first found to affect a range of lepidopteran insects (moths and butterflies), which are recognised worldwide as major agricultural pests on crops. Strains are now available which are toxic to coleopterans (beetles), dipterans (flies and mosquitos), lice, mites, protozoa and even nematodes but few details as to practical use are available (Ranjekar et al, 2003). It is these that makes Bt an effective insect pathogen. *Bacillus thuringiensis* δ-endotoxins are part of a large and still growing family of homologous proteins, more than 130 genes have been identified to date (De Maagd et al, 1999). Cry genes were first classified in different classes as cryI, cryII, cry III, cry IV based on protein structural homologies and host range. More recent analysis reveals that this classification is not necessarily based on homology or evolutionary relationships and a new nomenclature has been proposed (Jouanin et al, 1998). The subgroup and characteristics of cry genes were demonstrated in Table 1.2.

Table 1.2. Subgroups and characteristics of cry genes

Cry Gene	Crystal shape	Protein size-kDa	Insect activity
cry I [subgroups:A(a),A(b), A(c), B, C, D, E, F, G]	bipyramidal	130-138	Lepidoptera larvae
cry II [subgroups A, B, C]	cuboidal	69-71	Lepidoptera, Diptera
cry III [subgroups A, B, C]	flat/irregular	73-74	Coleoptera
cry IV [subgroups A, B, C, D]	bipyramidal	73-134	Diptera
cry V-IX	various	35-129	Various

The members of the cry gene family are grouped in subfamilies according to their specificity for members of insect families (De Maagd et al, 1999).

1.5.4. Mode of Action

The mechanism of action of the *B. thuringiensis* Cry proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry toxin to midgut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores (Schnepf et al, 1998). The δ-endotoxins, upon ingestion by the insect larva, are solubilized in the highly alkaline insect midgut into individual protoxins. The protoxins are activated by midgut proteases which cleave them into smaller polypeptides, the toxins. Activated Cry toxins have two known functions, receptor binding and ion channel activity. The activated toxin binds to specific receptors on the surface of epithelial cells in the midgut, inducing lesions that destroy the cells and lead to the death of the insect (Ranjekar et al, 2003, Jouanin et al, 1998). Lethality is believed to be due to destruction of the transmembrane potential with the subsequent osmotic lysis of cells lining the midgut (Aronson and Shai, 2001).

Differences in the extent of solubilization sometimes explain differences in the degree of toxicity among Cry proteins (Schnepf et al, 1998).

1.5.5. Commercially available GM maize events

A number of transgenic corn lines have been developed and approved in various countries. 17 GM corn events were used as food or feed and cultivated in the USA. However just 4 maize lines, Event 176, Mon810, Bt11 and T25, was approved by EU.

Furthermore, in Australia, New Zelland, South Africa, China, Japan, Argentina, Canada, Switzerland some GM maize lines have been approved and commercially available.

Most commonly used cry genes for the development of transgenic maize is cryIAb gene coding for a CryIAb protein (Figure 1.7).

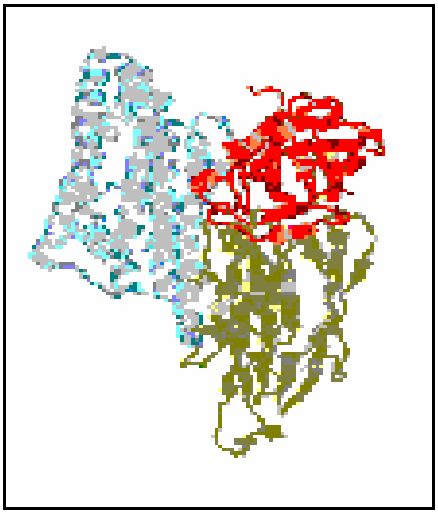


Figure 1.7. Cry 1Ab protein (De Maagd et al, 1999).

Cry1Ac gene has also been used so as to develop commercial insect resistant transgenic maize line (*Bt-Xtra***,** *DeKalBt* **/DBT418***)*. Additionally, cry 9c gene has been utilised to produce genetically modified insect resistant maize (Starlink**/ CBH-351**). Moreover cry3Bb1 has been used to develop Insect resistant GM mazie (*MaxGuard*, *YieldGard*, *Rootworm* / **Mon863)** insect resistant maize. Herbicide tolerance, insect resistance and male sterility are main characteristics in commercialized maize lines on the world food market.

Commercially available GM maize events were illustrated in the Table 1.3.

Table 1.3. Commercially available GM maize events (BATS Report, 2003).

Event and trademark	Notifier	Characteristics	Approval place
Event 176 *Knockout Maximizer NatureGard*	Novartis (formerly Ciba-Geigy)	Insect Resistant (cry1Ab)	EU, Australia, New Zelland, USA, South Africa, China, Japan, Argentina, Canada, Switzerland
Event 676, 678,680	Pioneer Hi-Bred	Male sterility	USA
Event B16	DeKalb, DeKalb Genetics	Herbicide	Canada, Japan, USA
Event Bt11 *Attribute YieldGard*	Novartis /Northrup King Co.	Insect Resistant (cry1Ab)	EU, Australia, New Zelland, USA, South Africa, China, Japan, Argentina, Canada, Switzerland
CBH-351 *Starlink*	AgrEvo, Inc.	Insect Resistant (cry9C)	Japan, USA
DBT418 *Bt-Xtra, DeKalBt*	DeKalb Genetics Corp.	Insect resistant, herbicide tolerance (cry1Ac)	Japan, USA, Argentina, Canada, Australia, New Zelland
DBT418-DK566	DeKalb Genetics Corp	Insect resistant, herbicide tolerance (cry1Ab)	Japan
DLL25-DK566	DeKalb, DeKalb Genetics	Herbicide tolerance	Japan
GA21 *Roundup Ready*	Monsanto	Herbicide tolerance	Australia, New Zelland, USA, South Japan, Argentina, Canada, Bulgaria, Russia

To be continued

Table 1.3. *(continued)*

Event and trademark	Notifier	Characteristics	Approval place
Mon810 *YieldGard*	Monsanto	Insect resistant (cry1Ab)	EU, Australia, New Zelland, USA, South Africa, China, Japan, Argentina, Canada, Switzerland
Mon832	Monsanto	Herbicide tolerance	Canada, USA
Mon863 *MaxGuard YieldGard Rootworm*	Monsanto	Insect resistant (cry3Bb1)	Canada, Japan, USA
MS3 *InVigor^{R} SeedLink™*	Plant Genetic Systems	Male sterility	Canada, USA
MS6	Aventis, formerly AgrEvo, US	Male sterility	USA
NK603 *Roundup Ready*	Monsanto	Herbicide tolerance	Australia, Canada, Japan, USA
T14,T25 *LibertyLink®*	AgrEvo, AgrEvo Canada Inc.	Herbicide tolerance	Australia, New Zelland, EU, USA, Argentina, Canada

1.5.6. Event: Bt11

Bt11 corn has been engineered to express the Cry1Ab delta–endotoxin insecticidal protein. This protein is known to be effective against certain lepidopteran insects, including European Corn Borer (ECB). ECB is a major corn pest that reduces yield by disrupting normal plant physiology and causing damage to the leaves, stalks and ears (BATS Report, 2003).

Vector pZO1502 derived from pUC18 has been used to engineer Bt11. Linear map of DNA construct used for transformation can bee seen in Figure 1.8.

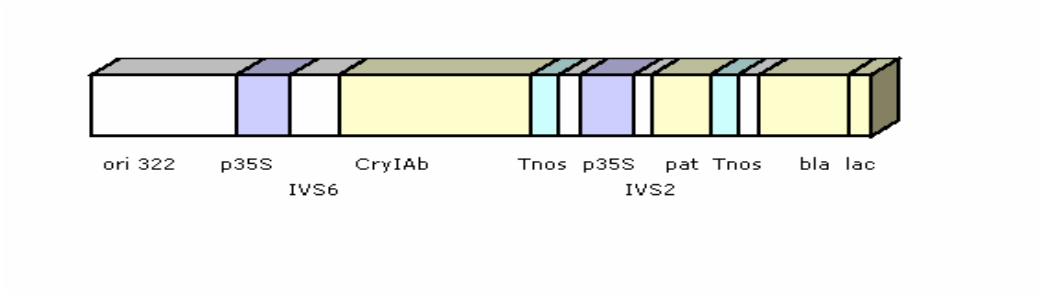


Figure 1.8. Sequence details of Bt11 (BATS, 2003)

Genetic elements located in the Bt11 gene construct was shown in the Table 1.4.

Table 1.4. Genetic elements located in Bt-11 gene construct (BATS, 2003)

Abbrevations	***Element Name***	**Size**
Ori322	Ori322	-
p-35S	Cauliflower 35S promoter	0.514 kb
IVS 6	Intervening seguence 6	0.472 kb
Cry1Ab	Cry1Ab delta endotoxin	1.84 kb
T-nos	Nos terminator	0.27 kb
Space	Space	-
p-35S	Cauliflower 35S promoter	0.42 kb
IVS 2	Intervening sequence 2	0.178 kb
PAT	Phosphinothricin acetyltransferase	0.558 kb
T-nos	Nos Terminator	0.22 kb
Space	Space	-
Bla	Beta-lactamase	-
Lac	Beta-galactosidase	-

Ori322 represents the *E.coli* origin of replication which ensures replication in *E.coli.* IVS2 and IVS6 introns derived from the maize gene adh1 (alcohol dehydrogenase-1S gene). PAT gene codes for a phosphinothricin acethyltransferase from *Streptomyces viridochromogenes*; homologue to bar. Bla stands for beta-lactamase gene; conveys resistance to beta-lactam antibiotics such as penicillin and ampicillin. Lac represents the beta-galactosidase. LacZ-alpha, the gene for the alpha region of beta-galactosidase under its bacterial promoter used for plasmid construct in *E. coli.* Additionally, LacZ is a partial lac1 repressor coding sequence, the lac promoter and a partial coding sequence fir beta-galactosidase (lacZ) protein. Cry1Ab delta endotoxin is a synthetic version of delta-endotoxin insecticidal protein derived from *Bacillus thuringiensis* subs. kurstaki strain HD-1. Nos terminator (T-nos) is the 3` non-translated region of the nopaline synthase gene derived from *Agrobacterium tumefaciens*. CaMV 35S promoter (p-35S) is a promoter derived from Cauliflower Mosaic Virus (BATS Report, 2003).

In the vector pZO1502, there is a deletion (of about 150 bp) in the junction between two gene cassettes and just at the beginning of the P-35S of pat cassette. Only one copy of the cry1Ab and pat genes are transferred into the plant genome. Additionally, the insert in the genome of the Bt11 corn contains an approximately 1.4 kb DNA of the vector sequence, upstream of the Cry1Ab cassette including ori 322 which orginates from *E.coli.* Ivs 2 and Ivs 6 are alcohol dehydrogenase gene (adh gene) from *Zea mays* (BATS Report, 2003).

It was suggested that genetically modified maize Bt11 did not present an increased risk to, or impact on interacting organisms, including humans, with the exception of specific lepidopteran insect species. Furthermore, Bt11 was not expected to have an impact on threatened or endangered lepidopteran species. Brandname(s) of the event Bt11 is YieldGard (BATS Report, 2003).

1.6. Genetic elements used in transgenic crops

The inserted genes into the currently approved transgenic plants are usually taken from other naturally occurring organisms, and have to undergo several

modifications before they can be effectively inserted into a plant genome and successfully expressed (BATS Report, 2003).

The genetic elements which have been used in particular cases may allow specific detection for the given transformation event. A screening method for detection of 26 approved transgenic crops was based on designing specific primers for promoter region originating from cauliflower mosaic virus (CaMV 35S) and terminator region originating from the nopaline synthase (nos) gene of *Agrobacterium tumefaciens.* (Hemmer, 1997, Pietsch et al, 1997). Mostly, foreign sequences are under the control of constitutive promoters such as Cauliflower Mosaic Virus (CaMV) 35S promoter. However in some cases tissue specific promoters or wound inducible promoters were also used (Jouanin, 1998). Many of the approved transgenic crops contain a copy of the constitutive 35S promoter (P-35S) from the CaMV or one of the derivatives of this promoter.

The most frequently used terminator in approved GM crops is nos terminator (T-nos), isolated from the nopaline synthase gene of *A. tumefacien*s. It is found in 37 products (BATS Report, 2003). Out of 66 surveyed transgenic crops, 62 of them contained at least one genetic sequence that was derived from these two organisms.

Another most frequently used transgene is nptII, originating from the *E. coli* transposon 5. This gene confers resistance to selected aminoglycoside antibiotics. In some cases nptII is under the control of bacterial regulatory elements, which does not allow expression in plants.

The *cry* genes are all synthetic and modified and in order to optimise gene expression in the host organism, truncated forms of the native genes are used. They are found in 20 transgenic products. The most frequently used *cry* genes are cry1Ab and cry3A present in 6 out of 20 products containing *cry* genes (BATS Report, 2003).

Figure 1.9 and Figure 1.10 shows the promoters and terminators used in commercially available GM crops.

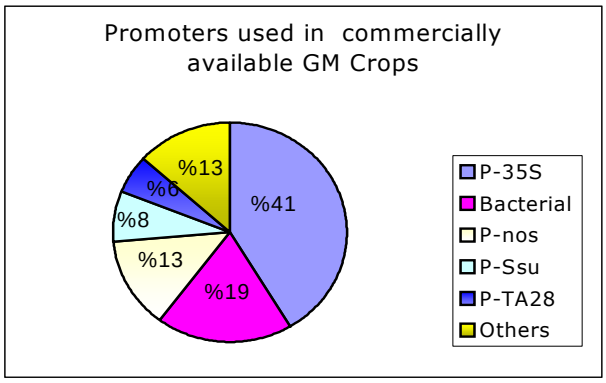


Figure 1.9. Frequently used promoters in commercialised GMOs (BATS Report, 2003)

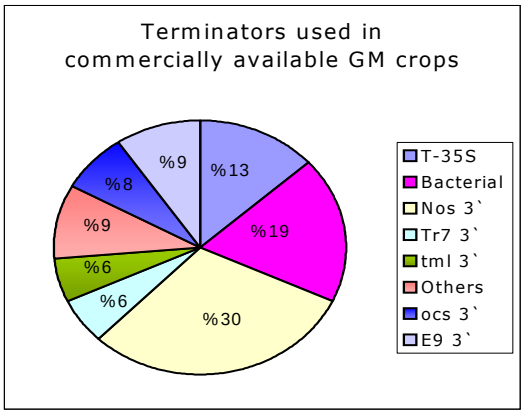


Figure 1.10. Frequently used terminators in commercialised GMOs (BATS Report, 2003)

1.7. Detection Methods of Genetically Engineered Foods

Raw materials and processed products derived from GM crops might be identified by testing for the presence of introduced DNA, or by detecting expressed novel proteins encoded by the genetic material (Farid, 2002). Numerous analytical

methods, both qualitative and quantitative, have been developed to determine the presence and the amount of genetically modified organisms (GMOs) in agricultural commodities, in raw agricultural materials and in processed and refined ingredients (Anklam et al, 2001).

It is important to differentiate that assessment of GMO content in samples can be divided into three different levels:

- **Detection (Screening of GMOs):** Detection is performed in order to gain a first insight into the composition of food and agricultural product (Anklam et al, 2001). The purpose of detection is to determine whether a sample contains GMOs. For this objective, a screening method can be used resulting in a positive/negative statement. The screening methods are usually based on the Polymerase Chain Reaction (PCR). Majority of GM plants have been transformed with constructs containing the CaMV 35S promoter or the *Agrobacterium tumefaciens* NOS terminator. Most commonly used cloning vectors are plasmids containing Kan^R antibiotics. Consequently, PCR methods targeting the P-35S, T-35S, T-Nos and nptII have wide applications for screening of the GMOs (Holst-Jensen et al, 2003).

- **Identification:** If there is a positive detection of GMOs, further analysis is required to discover which GMO it is and thus whether the GMO is approved by authorities. Identification reveals how many GMOs are present, and if so whether they are authorised within the EU or other countries with regard to their regulations. A prerequisite for the identification of GMOs is the availibility of detailed information on their molecular make-up (Holst-Jensen et al, 2003).

- **Quantification:** If a product has been shown to contain GMO(s), the next step is to assess compliance with the 1% threshold level (or the 0.3 or 0.5% level, respectively for seeds) by the determination of the exact amount of each of the GMOs present in the sample (Holst-Jensen et al, 2003, Anklam et al, 2001). Typically quantification is performed using quantitative competitive PCR or Real-time PCR.

The Experimental procedure utilised in the detection of GMOs can be summarised and observed in the Figure 1.11.

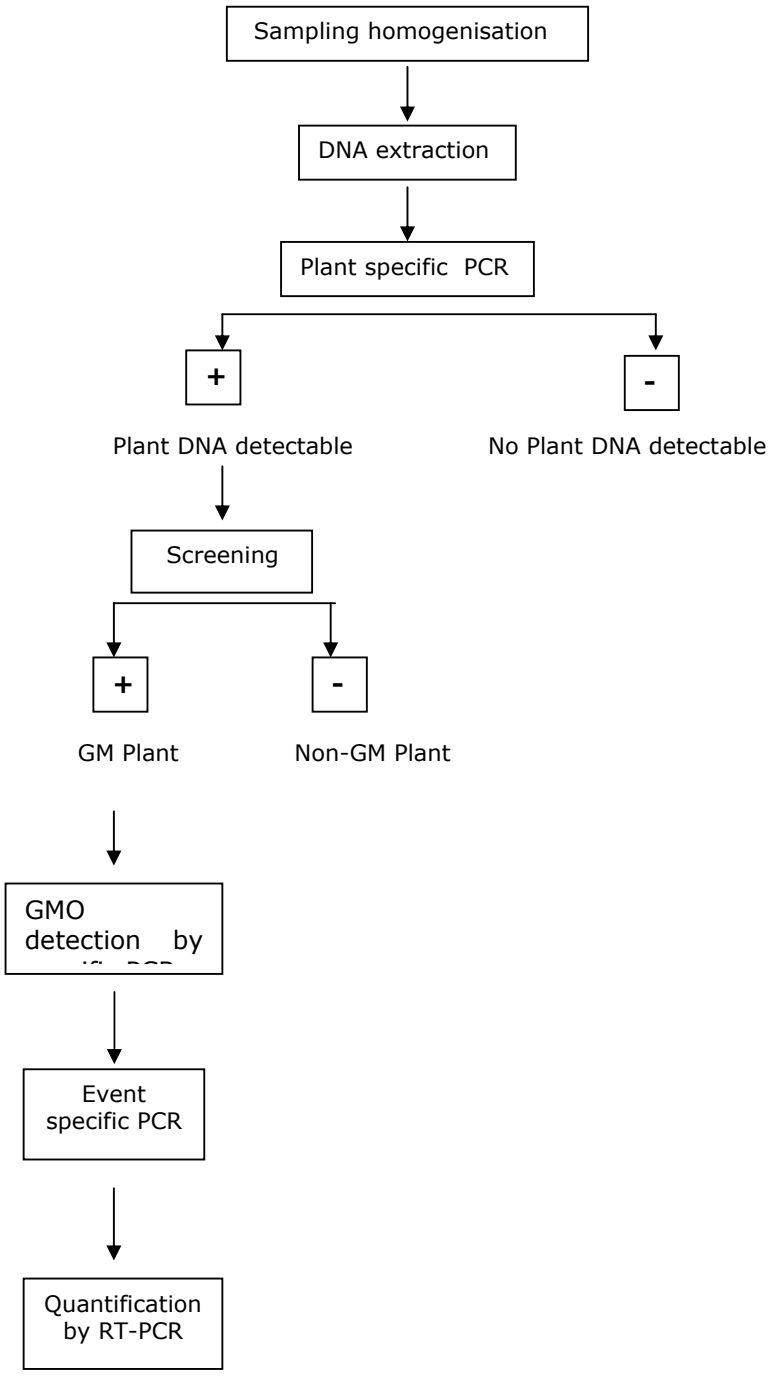


Figure 1.11. Experimental procedure of GMO detection

Methods for the identification of GMF can be divided into 2 categories. The first category is nucleotide-based amplification methods, which include the Polymerase Chain Reaction (PCR), fingerprinting techniques such as RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment length Polymorphism, and RAPD (Randomly Amplified Polymorphic DNA), probe hybridisation and microarray technology. The second category is protein-based methods including Lateral flow strip, Western-blot analysis, and Enzyme linked immunosorbent assay (ELISA). Every detection method has its own specificity and limitations (Lin et al, 2000, Chiueh et al, 2001).

1.7.1. DNA based methods

The DNA that has been engineered into a crop consists of several elements that govern its functioning. They are typically a promoter sequence, structural gene and the stop sequence for the gene. Analytical methods based on PCR technology are increasingly used for the detection of these DNA sequences associated with GMOs (Farid, 2002, Querci et al, 2002).

The principle of the PCR method is to multiply specific sequences of DNA, thanks to a pair of short DNA sequences (primers) that flank the region to be amplified making them detectable. The PCR method is based on the molecular structure of DNA. This highly sensitive method offers the advantage of detecting DNA molecules which are more thermostable than proteins (Gachet et al, 1999).

Using the PCR method to identify GM products, a primer is designed based on the regulatory sequence or structural gene in the inserted gene fragment. These designed primers possess some specific characteristics and can be used for product screening and product-specificity detection. The PCR products need to be further confirmed by the following methods: nucleic acid sequencing, endonuclease mapping, and probe hybridisation. The PCR method is not only used for identification of GM products, but also for quantification purposes. The methods based on PCR are not suitable for detection of highly processed foods because DNA fragments in foods could be broken into pieces . Nevertheless, PCR

is the most popular method used worldwide among the categories (Lin et al, 2000, Hupfer et al, 1997, Pietsch et al, 1997).

The PCR based GMO tests can be grouped into at least four category corresponding to their level of specificity . Each category corresponds to the composition of the DNA fragment that is amplified in the PCR. Category I represents the screening methods for detecting promoter and terminator sequences as 35S promoter and nos terminator. These screening methods can not be used to identify the GMO, since the presence of one of the screening targets does not necessarily imply presence of GMO-derived DNA. Category II represents the gene specific methods. he gene of interest may also be of a natural origin, but is often slightly modified, for example by truncation or altered codon usage. Consequently PCR methods targeting the gene of interest are more specific than category I methods. Category III is construct specific methods. Methods of category III, target junctions between adjacent elements of the gene construct, for example between the promoter and the gene of interest. By this method it will be possible to identify the GM source of the DNA. However, the full gene construct may have been transformed into more than one GMO, or may be used in future transformations, for example PV-ZMBK07 and PVZMG110 into the following GM maize: Mon809, Mon810, Mon832, Mon80100. Category IV is event specific PCR. The Only unique signature of transformation event is the junction at the integration locus between the recipient genome and the inserted DNA (Holst-Jensen et al, 2003). Screening methods cannot distinguish between GMOs containing the same insert at different loci. Moreover it is impossible to quantify each GMO in samples containing different transgenic events. To overcome these problems , a line, or transformation event specific PCR must be performed. Event specific primers amplify a fragment of a unique junction region between the inserted DNA and the plant DNA and therefore act as a unique identifiers. But data concerning these junctions are not always available or are confidential (Taverniers et al, 2001). Unfortunately, even the event specific methods have their limitations. When two GMOs are crossed (Two different GM maize such as T25 and Mon810), the resulting hybrid offspring may contain the genetic modifications including the signature of both events and will be indistinguishable from its two parents in a PCR test. This phenomenon is referred to as *gene stacking*. In the USA this type

of hybrid GMO is not regulated if both parent GMOs are authorised. In Europe, however the hybrid is treated as a new GMO and requires separate

authorisation. Not only does this create a problem with respect to identification, but it also creates a potential problem for quantitation (Holst-Jensen et al, 2003).

Another strategy for GMO identification recently discussed makes use of Amplified Fragment Lenght Polymorphism (AFLP), a DNA fingerprinting method, which has already been used successfully to discriminate between and identify plant varieties, including processed agricultural materials. Interestingly, AFLP has been investigated for its potential in the combined identification of the variety genotypes and monitoring of very low level of GM materials. 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 (Anklam et al, 2001, Bonfini et al, 2001). Southern Blot can also be used as GMO detection method.

Microarray based technology for detecting gene expression is currently under development. It has been developed in recent years for automated rapid screening of gene expression and sequence variation of large number of samples (Bonfini et al, 2001).

All DNA based methods are regarded as `*High-technology methods*` Among GMO detection methods, so far only PCR has found broad application in GMO detection as a generally accepted method for regulatory purposes.

Hubner and his co-workers emphasised that for the successful application of PCR, crucial factors are the following; the homogeneity of the sample to be analysed, performance of template isolation and purification in terms of yield and purity, standardized process for the estimation of concentrations of genomic DNA and all reagents used in the reactions. For the PCR itself, the crucial factors to be controlled are: set up of reactions, batch to batch variations of reagents, temperature time programmes used for the PCR amplification and the

performance of different types of hardware. The crucial factors for the post-PCR process is the detection of the amplification products of the PCR. The

tremendous sensitivity of PCR methods requires a careful and consequent separation of the three process in terms of hardware, laboratory space and sample handling (Hubner et al, (B), 1999).

The first identification method via PCR was performed to identify *Flavr Savr* ® tomato by Meyer and his co-workers (Meyer, 1995). In 1996, *Streptecoccus thermophilus* found in fermented milk products as yoghurt was examined by PCR. Moreover, recombinant *Lactobacillus sake* carrying foreign catalase gene was detected in raw sausage.

The first detection method for screening and identification of insect resistant maize was carried out by Hupfer and his co-workers via DNA based techniques (Hupfer et al, 1997). Event specific PCR was developed in order to demonstrate GM maize lines as Event 176 maize, Maximizer maize, Bt11 maize and Mon 810 (Chiueh et al, 2001, Lipp et al, 1999, Hupfer et al, 1999, Lin et al, 2000, Yamaguchi et al, 2003). To detect Bt11 maize line, Inverse PCR was improved and quantification was performed via Real-Time (Zimmerman et al, 2000). Methods for detection of seven lines of GM maize, such as Event 176, Bt11, T251, Mon 810, GA27, DLL25 were discussed (Matsuoka et al, 2002).

By nested PCR, detection procedures was carried out on soybeans (Pan and Shih, 2003), broccoli (Wolf et al, 2000) and maize (Zimmerman et al, 1998). Multiplex PCR was also performed for demonstrating presence of foreign genes in foodstuff (Tao et al, 2001 and Su et al, 2003). Windels and co-workers presented an anchored PCR strategy for the development of line specific GMO detection procedure (Windels et al, 2001, Windels et al, 2003). They also used AFLP, a DNA fingerprinting method for the detection of GMOs

Quantitative competitive PCR for the detection and quantification of the 35S promoter and Nos terminator was utilised (Hardegger et al, 1999, Tozzini et al, 2000, Wurz et al, 1999). In maize and soybean Real Time PCR was conducted by several scientists (Alary et al, 2002, Taverniers et al, 2001, Hernandez et al,

2001, Holck et al, 2002, Windels et al, 2003). Dual-competitive method was developed and utilised for processed products (Hupfer et al, 2000).

Furthermore, detection of genetic modification was conducted on processed foodstuffs. Owing to DNA degradation in processed foodstuffs, detection methods may not work. Processed foods were exposed to prolonged heat treatment, for instance autoclaving used in canning of maize, frying was performed for getting corn flakes, maize chips, pop corn. These process bring about the fragmentation and, hydrolysis of DNA. Manipulations on chemistry of DNA cause the PCR process unsuccessfull. Additionally, low pH treatments result in hydrolysis. Moreover addition of enzymes during processing can degrade DNA. Hupfer and his co-workers affected maize powder by low pH and high temperature for a period of time and conducted GMO detection (Hupfer et al, 1998). Another study was performed by Moser and co-workers (Moser et al, 1999). They achieve to detect foreign genes only in products not subjected to low pH or prolonged heat treatment. As a general rule, the lower the pH at which the product was treated, the lesser the chance to succeed with DNA based analytical methods.

In order to evaluate the possible transfer of plant DNA into two different farm animal species (cattle and poultry) with a special emphasis on detecting recombinant *Bacillus thuringiensis* toxin maize (Bt-maize) material in secondary animal products such as meat, eggs or milk, PCR was introduced (Einspanier et al, 2001).

1.7.2. Protein based methods

The protein based test method uses antibodies specific to the protein of interest (Querci et al, 2002). Immunoassay technologies with antibodies are ideal for qualitative and quantitative detection of many types of proteins in complex matrices when the target is known. Both monoclonal and polyclonal antibodies can be used. Western Blot, enzyme linked immunosorbant assay (ELISA) techniques, Lateral Flow Strip and other immunoassay formats using magnetic particles have been used (Farid, 2002).

ELISA uses one antibody to bind the specific protein, a second antibody to amplify the detection, and an antibody-conjugated to an enzyme whose product

generates a colour that can be easily visualised and quantified based on comparision of a standard curve of the protein of interest (Querci et al, 2002). A variation on ELISA, using strips rather than microtiter wells led to development of lateral flow strip technology (Farid, 2002). One of the major drawbacks of immunochemical assays is that their accuracy and precision can be adversely affected in a complex matrix, such as those found in many processed agricultural and food products. The possible causes for interference from the matrix have been attributed to non-specific interaction with the antibody by proteins surfactants, or phenolic compounds , antibody denaturating by fatty acids and the presence of endogenous phosphatases or enzyme inhibitors (Anklam et al, 2001). Furthermore, antibody test such as ELISA are much more laborious and time consuming to develop and to validate than nucleic acid tests. Moreover certain proteins may be expressed only in specific parts of the plant like leaves, beans, pollen and stems, the whole genetic information is present everywhere in plant, because the same genes are present in each cell of the plant (Gachet et al, 1999).

The detection of enzymatic activities method is not recommended for processed foods, where proteins may be denaturalised (Lin et al, 2000). Detection or measurement may be rendered difficult by low levels of expression of transgenic proteins, the degradation associated with thermal treatments or by a poor antibody affinity of the commercially available source of antibodies (Anklam et al, 2001). Additionally ELISA is less sensitive than PCR, therefore less susceptible than PCR to false positive caused by the minor levels of contamination. Moreover it can not discriminate between different expression patterns and modes among different transgenic events that express similar protein characteristics (Querci et al, 2002).

Among the methods that can be used in laboratory GMO monitoring, the PCR-ELISA offers many advantages. It detects and identifies PCR products by hybridisation in solution with one or two internal probes (Petit et al, 2003).

In addition to protein and DNA-based methods employing western blots, enzyme-linked immunosorbant assay, lateral flow strips, Southern blots, qualitative-, quantitative-, real-time- and limiting dilution-PCR methods,

providing that the information on modified gene sequences is not available, another approaches, such as near-infrared spectrometry, SDS-PAGE, 2D-Gel electrophoresis might tackle the problem of detection of non-approved Genetically Modified (GM) foods (Pan, 2002 and Cellini et al, 2004). Proteomics approaches are so crucial to our understanding of development, structure and metabolism and may be a promising method for the detection and understanding of unintended effects in GM food crops in the future (Cellini et al, 2004).

1.8. Quantitation methods

A crucial aspects of analysis of GMOs in food is quantitation, because maximum limits of GMOs in foods are basis for labelling in the European Union. Therefore more quantitative PCR approaches are needed (Farid, 2002). Quantitative-competitive PCR and Real-Time PCR have been developed which address the problems of establishing a relationship between the concentration of target DNA and the amount of PCR product generated by amplification. (Weighardt, 2002).

1.8.1. Quantitative-competitive PCR

Quantitation is done by comparision of the amount of end product, that is when the PCR reaction is completed. Two target sequences with very similar features and amplifiability are co-amplified in a single reaction tube. Competitive PCR requires development of suitable competitor molecules (Holst-Jensen et al, 2003). In this case, the standard is known amount of synthetic DNA added. The result can only indicate a value below, equal to or above a defined standard concentration.

1.8.2. Real time PCR

A more accurate and currently more widely used quantitative PCR methodology is represented by real time PCR. In contrast to the end-point determinations, real time PCR systems monitor the reaction as it actually occurs in real time (Mazzara and Querci, 2002). A unique features of this PCR technique that

the amplification of the target DNA sequences can be followed during the whole reaction by indirect monitoring of the product formation. Therefore the conventional PCR reaction has to be adapted in order to generate a constant measurable signal, whose intensity is directly related to the amount of product formed. Real time detection strategies rely on continuous measurements of the increasments in the fluorescence generated during the PCR (Anklam et al, 2001). Fluorescence signal corresponding to increased amount of amplification product can be measured and visualised on a computer screen. Software can immediately convert the signal into quantitative estimates (Holst-Jensen et al, 2003).

Various chemistries have been developed for monitoring the signals. These are intercalating dyes as EtBr and SYBR Green Dye I and hybridisation probes as TaqMan probes, FRET probes, molecular beacons and scorpions. The problem of amplicon fluorescent detection specificity has been overcome using sequence specific probes with a fluorescent labelling inside the PCR primer pairs. For energy transfer, donor and acceptor molecules should be in close proximity. If the donor and acceptor fluorophor are in close proximity to each other, excitation of the donor by blue light results in energy transfer to the acceptor which can then emit of longer wavelenght. Energy transfer can be converted to a measurable signal by software (Querci et al, 2002).

GMO content of a sample is a percentage of the amount of genetically modified material in the total material amount. In order to determine this value in a real time PCR based system , it is necessary to measure an endogenous reference gene for use as a normaliser as well as the GMO specific target DNA sequences. The reference gene should be chosen in order to be species specific, being present as a single copy per haploid genome, being stably represented as in different lines of the same species. Therefore, in Real-Time PCR analysis, one PCR system was designed to detect a GMO specific target DNA sequences. A second PCR system was designed to detect an endogenous reference

sequences. The reference specific quantification and GMO specific quantification occurs in the same PCR run not in different run, to avoid a possible statistical fluctations between different experiments (Querci et al, 2002).

One problem in relative quantification arises from legal definition of percentage of GMO content. Since it can be assumed as the weight of the pure modified ingredient over the total weight of the pure ingredient (e.g. weight of GM maize over total weight of the entire maize contained in the sample). From the practical point of view, it is possible to assume that GMO percentage is calculated as the number of modified genomes over the total number of the genomes of given species contained in one sample (Querci et al, 2002).

1.9. Limit of quantitation (LOQ)

It can be calculated that if samples are distributed in a binomial manner, 299 maize kernels are needed to detect 1% GMO with a confidence of 95% probability. If the DNA is extracted from e.g. homogenised maize chips, it might well happen that in the final reaction mixture for DNA amplification, total genome copy number is below the critical practical size and hence , that the relationship between sample size at the beginning and at the end of the experiment has been lost (Bonfini et al, 2001).

DNA amount in the unreplicated, haploid nuclear genome of an organisms is referred to as its C value. *Zea mays ssp. mays*, for instance, has ten chromosomes in its haploid nuclear complement. The DNA content of the unreplicated haploid complement (n=10) is known as 1C value.

Significant intra-specific variations in nuclear DNA amount occurs in *Zea mays ssp. mays*, so a DNA C value cannot be necessarily correct for all lines of this species. The estimated 1C total nuclear DNA value ranges from about 2,45 pg (2,364 Mb) to 3,35pg (3.233 Mb). In 1994, over 120 estimates of DNA C values for more than 100 genotypes of *Zea mays ssp. mays* from 25 sources were known. Based on the 1C values 100 ng of DNA may contain approximately 3.8 x 10^4 copies of maize genome. This corresponds to 38 genome copies for maize if only 0.1% of 100ng of DNA is of GMO origin. Consequently theoretical detection

limit (which is equivalent to one copy of unreplicated haploid genome) in a 100 ng DNA reaction is 0.003% in the case of maize (Bonfini et al, 2001).

1.10. Legislation of Genetically Modified Foods (GMFs)

All GM varieties have been formally assessed for safety by the competent authorities in the countries in which they are grown and most countries have specific legislation requiring such an assessment to be made and detailing the approach to be taken. Virtually all GM crops assessed for safety to date have both food and feed use. As the greatest concerns have related to human exposure, not surprisingly the emphasis of most regulatory authorities has been placed on food use. Safety for animals, while not ignored, has been a lesser concern and generally, and not unreasonably, been assumed to the follow automatically from the food safety assessment (Aumaitre et al, 2002).

1.10.1. Regulation in the European Union (EU)

There is a tremendous need for following the biosafety regulations, more responsible public debate and social attitude (Sharma et al, 2000). The use of Genetically Modified Organisms (GMOs), their release into the environment, cultivation, importation, and particularly their utilisation as food or food ingredients, is regulated in the European Union by a set of strict procedures (Querci et al, 2002). According to EC novel Food Regulation 258/97 and regulation 1139/98/EC and 49/2000, foods require labelling if more than 1% of any ingredient originates from a GMO (Holck et al, 2002, Commission Regulation (EC), No 1139/98 and Novel Food Regulatin (EC), No 258/1997). The Council Regulation 1139/98/EC of 26 May 1998 provides the basis for the labelling of foodstuffs derived from GM maize and GM soybeans (Meyer, 1999 and Moseley, 2002). More recently, the so called threshold regulations 49/2000/EEC was approved that specifies that foodstuff must be subject to labelling where material derived from these genetically modified organisms is present in food ingredients in a proportion above 1% of the food ingredients

individually considered. (Hardegger et al, 1999 and Commission Regulation (EC), No 1139/98) Norway and Switzerland which are not members of EC, both demand the labelling of GMOs in their food laws. In Norway labelling limit of 2% GMOs in food has already been set (Hardegger et al, 1999) whereas in Switzerland it has been set as 1% (Hubner et al, (A), 1999).

Although legislation at the national level as well as that of the EU generally permits release and commercializations of GMOs only 10 plant/event have been registered by 2002. Three of them are maize (Bt11, Bt176, Mon 810). Two maize events (Bt11 and Mon810) cannot be used for large scale commercialization because national variety registirations were refused in most countries (Saeglitz and Batsch, 2003). Approved maize lines can be seen in the Table 1.5. In February 1999, a request was submitted under Regulation (EC) 258/97 for placing sweetcorn from GM maize line Bt11 on the market for food use (fresh or processed). On 17 April 2002, the Scientific Committee on Food gave its opinion that Bt11 sweetcorn is as safe for human food use as its conventional counterparts. Since, 19 may 2004, the ban has been lifted on Bt11. France, Austria, Luxembourg, Denmark, Portugal, Greece voted against the approval in the 26 April Agriculture Council (in national level). Bans are permitted under the Novel Food Regulation. (http://www.gene.ch, 03-03-2004) History of regulations were represented in Appendix H (Lee and Carson, 2004).

Table 1.5. Approved GM maize events in EU

Maize Lines	Notifier	Main Traits	Commission decision
MON 810	Monsanto	Expression of CryIAb gene	94/294/EC
Bt11	Novartis	Expression of cryIAb gene and tolerance to Glufosinate ammonium	98/292/EC
T25	AgrEvo	Tolerance to glufosinate ammonium	98/293/EC
Bt176	Ciba Geigy	Expression of cryIAb gene and tolerance to Glufosinate ammonium	97/98/EC

1.10.2. Regulation in the USA

A large number of GMOs are approved for use in the USA and are grown on a large and increasing scale (Holck et al, 2002). In the USA, there is no obligation for labelling of GM foods. The USDA, FDA and EPA are involved in regulating GMOs (Thomson, 2003 and Harlander, 2002).

1.10.3. Regulations in Japan

Thresholds have also been introduced by other countries. In Japan, a threshold of 5% for frequently used GM crops like soybean and maize was implemented. Aside from labelling, The Ministry of Health and Welfare announced that health testing of GM crops is required (Hino, 2002).

1.10.4. Regulation in developing countries

The United Nations Environmental Agency (UNEP) and the Global Environmental Fund (GEF) have launched a project aimed at enabling 100 countries to develop National Biotechnology Framework by June, 2004 (Thomson, 2003).

1.10.5. Regulation in Turkey

Cartagena Biosafety Protocol (CBP) which seeks to protect biological diversity from the potential risks resulting from modern biotechnology has been signed by Turkey on 24-05-2000. Food law (No5179) which was focusing on controlling of all commercialised foods was implemented on 27 May 2004. New food law has an article about preparation of GM Food regulations.

Though there are some regulations about field trials of GM crops, till now, genetically modified foods has never been the object of specific regulation in Turkey. A new law dealing with GM food and feed is still under discussion and in progress (www. tagem.gov.tr, 21-08-2004). Furthermore, there is no accreditated laboratory for GMO detection.

Regulatory oversight, or safety assessment, in the food sector should be focused on such matters as labelling, export, import, cultivation, development and consumption of novel foods or novel food ingredients derived from Genetically Modified crops.

History of regulations on GM foods was summarised and demonstrated in Figure 1.12.

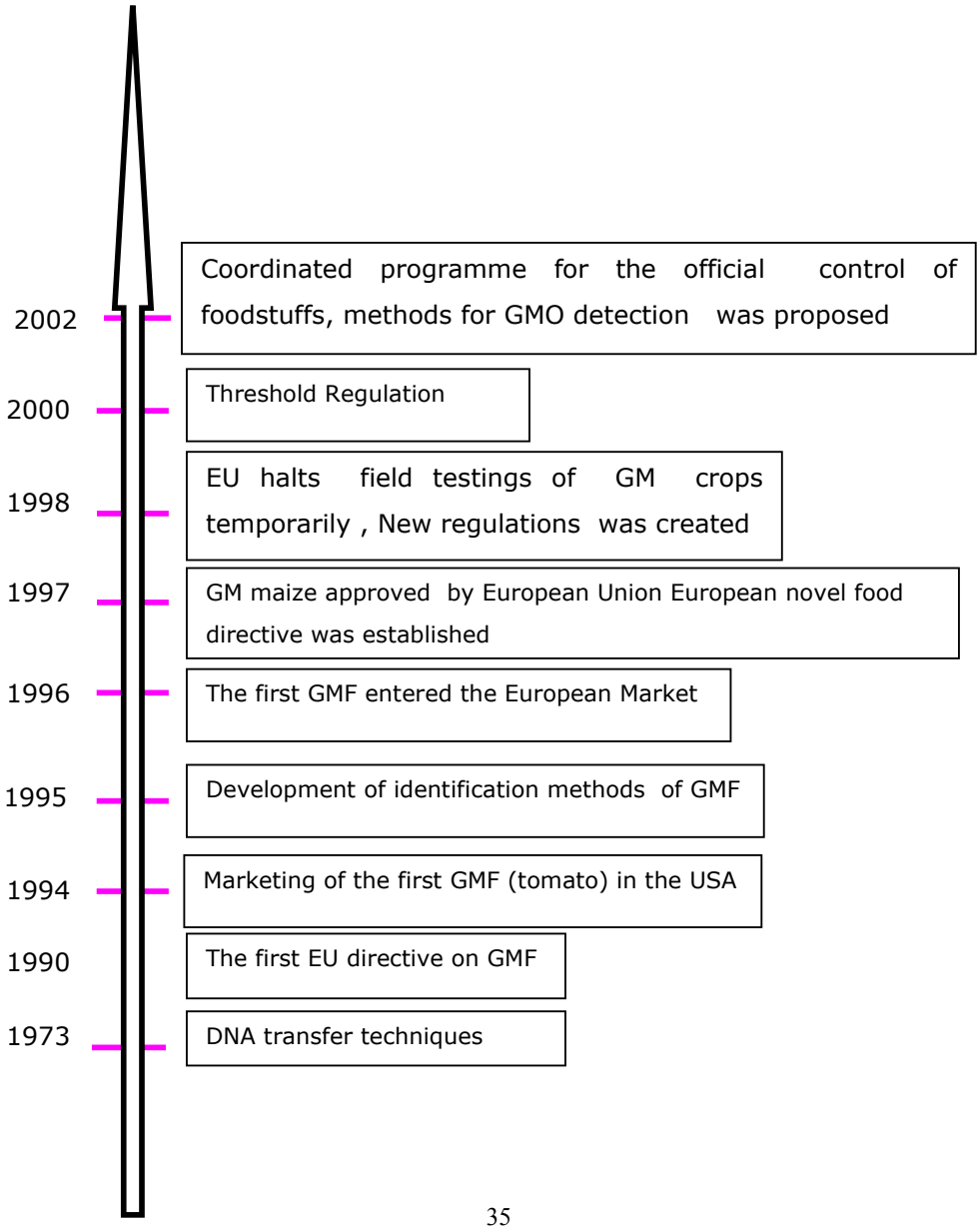


Figure 1.12. Timeline of GM Foods (http://www.nutrition.org.uk/flairflow/booklets/FF4GMandFood.pdf, 21-05-2004).

1.11. Benefits of GM Crops

Genetic engineering was aimed at benefiting mankind and was utilized in the first generation of so-called GM crops to provide growers with complementary and sometimes alternative crop management solutions to pesticides (Malarkey, 2003).

Supporters of genetic engineering of foods, including members of private industries, food technologists, food processors, distributors, retailers, scientists, nutritionists, some consumers, U.S. farmers, and regulatory agencies, advocates for the world's poor and hungry people, as well as proponents of the Green Revolution, think that because genetic engineering techniques have recently become simplified, the methods can be applied to the large-scale production of food and drugs needed by the ever-growing world population. In addition, genetic engineering may lead to faster growing, disease-, weather-, and pest-resistant crops, herbicide tolerant crops, as well as tastier, safer, more convenient, more nutritious, longer-lasting and health-enhancing foods. Proponents of GM foods believe that prospects for benefiting humanity are almost limitless, and that GM can potentially solve critical problems of world

1953 Double helix structure of DNA

agriculture, health, and ecology (Uzogara, 2000).

❖ **Impact of GM Crops to developing countries**

Development of GM foods is motivated by two global forces which are mounting population pressure and rising quality of life expectations (Garza and Stover, 2003). There is a continuing, need to increase the output of world agriculture if the demands of a rising world population are to be met (Hilder and Boulter, 1999). Despite anticipated reductions in growth during this century, the world`s population in 2050 is expected to have increased by 132-182%, that is to between 7.9 and 10.9 billion.

Increasement of population is especially observed in less economically developed countries as Bangladesh, India, Pakistan and Nigeria. It is projected that 75% of the worlds population will reside in the worlds less economically

developed countries by mid-century. Many expect that these population pressures will exacerbate food insecurity and micronutrient deficiencies, unless issues of food availability and accessibility are addressed in an anticipatory manner. Rising quality of life expectations represent the second force that motivates GM Foods (Garza and Stover, 2003). In poor countries where the malnutrition and ill health are frequent consumers typically spend 70% of their incomes on food and diets consist primarily of staple foods which lack the vitamins, minerals and very likely, other food components necessary to sustain food health and minimise the risk of adult onset diet related chronic diseases (Bouis et al, 2003).

GM crops will be an important tool to alleviate the malnutrition and starvation steming from increasing population of world. Golden rice is one of the most important example in this way. Beta-carotene dense rices so named as *"Golden Rice"* helps alleviate the malnutrition. Researches have demonstrated that many children and adults suffer more from a lack of essential vitamins and minerals in their diets than from a lack of calories. These deficiencies bring about poor eyesight, impaired cognitive development, physical growth, more frequent and severe bouts of illness, and high mortality. Since there is a great physiological need for iron, more than 40% of women and preschool children who are consuming conventional rice in Asia are anameic. Golden Rice is found as a way to increase the intake of iron (Bouis et al, 2003).

- **Impact of GM Crops to yield and agricultural practice**

Loses due to pests and diseases have been estimated at 37% of the agricultural production world-wide with 13% due to insects. Crop losses from insect pests can be staggering, resulting in devastating financial loss for farmers and starvation in developing countries. Farmers typically use many tons of chemical pesticides annually. Consumers do not wish to eat food that has been treated

with pesticides because of potential health hazards, and run-off of agricultural wastes from excessive use of pesticides and fertilizers can poison the water supply and cause harm to the environment. In order to overcome this problem, transgenic crops were developed (Jouanin et al, 1998).

There is also huge economical impact of GMF. Growers sustain billions of dollars in crop loss or reduced yield due to pests which have the potential to be controlled by Cry proteins. In cases such as European Corn Borer, stalk damage caused by second generation borers which have entered the inside of the corn stalk is difficult to control with externally applied pesticides. In addition, important chemical insecticides, are loosing their effectiveness due to the onset of pest resistance (Betz et al, 2000).

For some crops, it is not cost-effective to remove weeds by physical means such as tilling, so farmers will often spray their fields with large quantities of different herbicides (weed-killer) to destroy weeds, a time-consuming and expensive process, that requires care so that the herbicide doesn't harm the crop plant or the environment. Crop plants genetically-engineered to be resistant to one very powerful herbicide could help prevent environmental damage by reducing the amount of herbicides needed. Apart from insect resistant and herbicide tolerance plants, virus and disease resistant crops has a huge impact on yield and agricultural practice. There are many viruses, fungi and bacteria that cause plant diseases. Plant biologists are working to create plants with genetically-engineered resistance to these diseases. The USA, Canada, Japan, Argentina, China, Brazil and South Africa top the list of countries planting GMOs (Thomson, 2003).

1.12. Criticisms against GM Foods

The release of transgenic plants has aroused debates about several aspects of the environmental and human risks that could result from the introduction of genetically modified crops (Azevedo and Araujo, 2003). Some of these critics include consumer and health advocacy groups, grain importers from Europe, organic farmers, public interest groups, some concerned scientists and environmentalists. Others are politicians, trade protectionists, ethicists, human rights, animal rights and religious rights groups, while the rest are chefs, food

producers, and food advocacy groups. These critics believe that applying GM techniques to human food production could have several adverse consequences. For the critics, safety, ethical, religious, and environmental concerns far outweigh the interest in improved food quality, increased food production, and improved agriculture brought about by GM techniques. These critics, especially those in EU countries, view GM as a suspect new technology that threatens world agriculture, health and ecology, hence they sometimes label GM foods with names such as '*Frankenfood*,' '*Farmageddon*' etc (Uzogara, 2000).

The process of genetic engineering creates risk and uncertainty in a number of ways. By transferring genetic information, genetic engineering can destabilize the way of DNA replicates, transcribes and recombines. This alteration of DNA sequences may have unintended and unexpected effects on the cellular processes of the recipient organism. This uncertainty is further compounded by the imprecise techniques used for inserting DNA, which prevents scientists from determining which regulatory functions might be affected (Akhter et al, 2001).

Table 1.6. Implications of genetic manipulation and human health (Akhter et al, 2001).

Implications of genetic manipulation and human health.
Inserted gene may have adverse effects
May code for proteins that are toxic for human
May produce an allergenic reaction
May cause activation of previously silent genes
May be transferred to other organisms
Consumption of GMO may alter balance of existing micro-organisms in the gut

As a result of altered regulatory functions, genetically modified organisms (GMOs) may exhibit increased allergenic tendencies, toxicity, or altered nutritional value. They may also exhibit mutations, which are errors that can

occur in the sequences or reading of the DNA within the cell. Altering regulatory functions may create new components or alter levels of existing components of an organism. One concern often expressed is that this may create antibiotic-resistant bacteria. Such risks are further aggravated when a GMO is released in

the environment. The interaction of GMOs with humans or natural ecosystems cannot be anticipated or tested before commercial release. This complexity makes it difficult to determine the short- and long-term effects of genetic modification. Food crops that are engineered to be resistant to pesticides and herbicides perpetuate reliance on these chemicals, promote environmentally harmful farming methods, and may encourage pests and insects to develop pesticide resistance. It can also threaten to transfer resistance to wild species, with implications for biodiversity and ecosystem integrity (Akhter et al, 2001).

1.12.1. Environmental hazards

Ecological risks might be classified into three groups**.**

- **Introgression of transgenes into weedy relatives**

One potential disadvantage of GM crops is that they might become, or generate by cross-pollination, superweeds that are difficult or even impossible to kill with herbicides normally used on the crop, thus causing environmental damage. Concerns have been expressed about the possibility that a gene coding for a trait such as herbicide resistance could pass from a crop to a weedy relative This is a concern that has been expressed particularly in the case of oilseed rape or canola (*Brassica napus*) that is resistant to herbicides that contain glyphosate as their active ingredient. *B. napus* does have some weedy relatives that it can be cross pollinate (Thomson, 2003).

One country in which out-crossing of pollen from GM maize to wild species could occur is Mexico, which is home to teosinte, the progenitor of modern maize. A recent paper in Nature by Quist and Chapela (2001) reported that the appearance of the Bt gene in landraces of maize in Mexico was due to the transfer of pollen from Bt maize in the USA. (Thomson, 2003 and Stewart et al, 2003). What is much more likely is that Bt maize seeds were introduced into

Mexico from maize farms in the USA. Cross pollination by this Bt maize of Mexican landraces could really occur. Events such as this are probably inevitable. The Quist and Chapela paper raised concerns about the potential effects of the introduction of transgenes on the genetic diversity of crop landraces and wild relatives in areas of crop origin and diversification. However the paper was the subject of considerable criticism (Thomson, 2003).

- **Impact on non-target species**

Another environmental impact of GMF crops that has been studied in considerable detail is the effect of pollen containing the Bt gene on non-target insects (Thomson, 2003). A highly publicised example of the effects of Bt pollen on non-target insects was the case of Monarch butterfly (*Danaus plexippus*). Larvae feeding on milkweed dusted with GM corn pollen were found to have reduced survival. Because the host plant of this butterfly is found in the victinity of corn fields, certain questions are arised (Hails, 2000). Laboratory-based studies showed that larvae feeding on milkweed (*Asclepias syriaca*) leaves covered with pollen from Bt-expressing maize suffered higher mortality rates than larvae feeding on milkweed covered with pollen from non-GM maize (Thomson, 2003).

- **Horizontal transfer of antibiotic resistance genes**

The horizontal transfer of genes from bacteria to plants is well known, a typical example being the transfer of DNA from *Agrobacterium tumefaciens.* Persistance of transgenic DNA from plants in the soil followed by the bacterial genetic transformation is one way in which transfer of DNA from plants to microorganisms may occur. The process of plant transformation requires that transgenic callus, and later developing plants, can be selected from the background of non-transgenics (Thomson, 2003). In some cases, the gene of interest is linked to a selectable marker gene which confers antibiotic resistance. Such resistance, if transferred to pathogenic microorganisms, might exacerbate the problem of resistant strains of bacteria. For example, an American cottonseed construct contains an *aad* gene, which confers resistance to streptomycin and spectinomycin. This has not been granted approval for marketing in the European Union. Spectinomycin is a useful drug used in the

therapy of infections caused by *Neisseria gonorrhoeae*, particularly in pregnant women, where the use of other antibiotics may be contra-indicated. Therapeutic options for this group are limited. Already a clinically significant resistance to third generation cephalosporins used in the treatment of gonorrhoea and other sexually transmitted diseases has been reported world-wide. This is of particular relevance when considering GM crops that carry the bla_{TEM} gene from which cephalosporin resistance has evolved. Horizontal gene transfer across related species and even unrelated organisms is a well-documented phenomenon. Gene exchange mechanisms include transduction, transformation and conjugation, with the latter being the mechanism with the broadest host range of transfer. The implications of this in the context of biosafety are far reaching. One particularly important example of this is the case of animal feedstuffs, where material from transgenic plants encounters bacteria in the ruminant gut. It is not clear whether the transfer of such genes from transgenic plant to microbe can be completely excluded; for example, it has been shown that oral bacteria can be transformed by naked DNA in human saliva. Moreover, DNA from an M13 mp18 phage ingested by mice was detected in their gastrointestinal tract as fragments of up to 1 kb in length. It was able to penetrate the intestinal wall into the nuclei of spleen and liver, where it was found to be covalently linked to the host DNA. There is evidence to suggest that foreign DNA originating from daily intake of food may be covalently linked to the host DNA. The likely consequences of such genetic recombinations for mutagenesis have not been investigated. However, it was observed in a cell-free system that specific patterns of de novo methylation of foreign DNA occur as a result of insertion of foreign DNA into the mammalian genome, thus triggering extensive changes in cellular DNA methylation patterns at sites far away from the locus of insertional recombination. These alterations may contribute to the potential of foreign DNA to induce oncogenesis. Therefore one aspect of the current debate on the safety of GM food is to know what conditions are necessary in installations preparing animal feeds to prevent gene transmission, i.e. what conditions of food processing would ensure sufficient disruption of DNA. Food ingredients are likely, in the future, to originate from a variety of GM sources containing a number of different transgenes. GM maize incorporating a L-lactamase gene which confers resistance to penicillin has, for example, generated great interest in view of the small but finite possibility of the

genetic material being transferred to potentially pathogenic microflora, thus rendering them antibiotic resistant. Concern has been expressed over the possibility that this gene might be transferred horizontally from a GM crop to bacteria, thereby increasing levels of antibiotic resistance (Chiter, et al, 2000).

Many countries are requiring that developers phase out the use of Antibiotic Resistance Markers (ARM) from GM crops. One strategy is to use techniques that allow the antibiotic resistance gene to be removed after the initial selection of transformed cells. In the *Cre/loxP* system, for example the transgenic cells are further transformed with DNA carrying the cre gene, which codes for a recombinase enzyme. The recombinase enzyme removes the antibiotic resistance gene that had originally been inserted between the *lox* sites in the original transformation event; however, the DNA carrying the *cre* gene is not incorporated into the plant genome so will be lost (Thomson, 2003). Moreover, transposable element based systems, co-transformation systems intrachromosomal recombination systems and MAT-vector systems were also utilised for removing ARMs (Scutt et al, 2002). Another strategy is to use selectable marker genes that yield plants resistant to compounds that are toxic but not antibiotic. One such example is the *manA* gene encoding phosphomannose isomerase. Most plant cells cannot grow on media containing the sugar mannose-6-phosphate as a sole carbon source; however, transgenic plants expressing the *manA* gene are able to do so. A similar strategy uses the *xy1A* gene, coding for xylose isomerase, to allow plant cells to survive on the sugar, xylose (Thomson, 2003).

1.12.2. Human Health Risks

In some ways, transgenic technology for crops awaiting to call to commerce still has a black box quality. Genes for given characteristics have been cloned and in many instances, the activity of native protein has only been demonstrated by the change brought about *in planta*. Therefore its potential of becoming an allergen remains unknown (McKeon, 2003).

The main concerns about adverse effects of GM crops on health are the transfer of antibiotic resistance, toxicity and allergenicity (Lack, 2002).

An example of transfer of a gene from an allergenic source that codes for an allergenic protein is that of the Brazil nut (*Bertholetta excelsa*). 2S albumin expressed in soybean. This protein is rich in methionine and would therefore increase the nutritive value of soybeans for animal feed. However, it was found that the newly expressed protein in soybean was reactive towards sera from patients who were allergic to Brazil nut. This observation blocked further development of the GM product (Kuiper and Kleter, 2003).

The type of biological action that the newly introduced protein has directs further toxicity testing in mammals. Studies have been undertaken on the binding of Cry1Ab5 and Cry9c proteins to tissues of gastrointestinal tract of rodents and primates, including humans. There is no evidence for these proteins in mammalian tissues, or for amino acid sequence homology with known protein toxins/allergens. Single and repeated dose toxicity studies of Cry1Ab5 and Cry9C did not indicate toxic effects in the rat, and histopathological analysis did not show binding of the Cry proteins to the intestinal epithelium of rodents or tissues of other mammals. In contrast to Cry1Ab5, Cry9C showed resistance to proteolysis under simulated human gastric conditions. Since there seems to be a relationship between protein resistance to proteolysis and allergenic potential, this characteristic needs further investigations been reviewed (Kuiper and Kleter 2003). Starlink episode provides the backdrop for concerns about industrial crops entering to food chain (McKeon, 2003). However maize containing Cry9C (Starlink yellow maize) was restricted to animal feed use, traces of Starlink maize were found in taco shells and subsequently a number of consumers reported allergic reactions after eating maize products. Cry 9C protein might be considered as a potential allergen because of the resistance to gastric proteolytic degradation and to heat acid treatment, the capacity to induce a positive IgE response.

1.12.3. Alterations of host metabolic pathways of active substances

Concern has been expressed about the potential for pleiotropic and insertional mutagenic effects. The former term refers to the situation where a single gene

causes multiple changes in the host phenotype and the latter to the situation

where the insertion of the new gene induces changes in the expression of other genes. Such changes due to random insertion might cause the silencing of genes, changes in their level of expression or, potentially, the turning on of existing genes that were not previously being expressed. Pleiotropic effects could be manifested as unexpected new metabolic reactions arising from the activity of the inserted gene product on existing substrates or as changes in flow rates through normal metabolic pathways (Malarkey, 2003).

1.13. Risk assessment of Genetically Modified Foods (GMFs)

Safety assessment of GMFs based on the concept of `*Substantial Equivalance*`. The concept of substantial equivalance embodies the idea that conventional foods can serve as comparators for the properties of GMFs, since conventional foods are considered as safe, based on a history of safe use (Kuiper and Kleter, 2003). *Substantial Equivalance* uses a comparative approach to reveal both intended and unintended differences between GM food and its conventional counterpart (Gasson and Burke, 2001). Comparison takes the agronomical, morphological, genetic and compositional properties of the GMF and traditionally produced food, and establishes the degree of equivalence between the GMF and the traditional counter part. Application of the concept of substantial equivalence does not lead to the establishment of the absolute safety of GMF, but it does provide insight into whether the GMF can be considered as safe as the conventional counterpart. Testing should be performed on GM plants and their comparators grown under identical conditions as environmental conditions may lead to phenotypic and genotypic differences not related to the specific transformation process (Kuiper and Kleter, 2003). The safety assessment strategies for GMFs should be designed and carried out on a case-by-case basis, according to the degree of equivalence with the conventional counterpart. Before embarking on toxicity studies of novel proteins, molecular, biochemical, structural and functional characterisations should include degradation under conditions of ingestion, processing and storage; biological response, immunological activity and sequence homology with proteins known to cause adverse effects (Kuiper and Kleter, 2003).

1.14. Public perception and attitudes about GM crops

At the consumer level, there are worries about the future safety of the gene technology used in crop development. Consumer concerns across the globe are not uniform (Moseley, 1999). Increased consumer concern for GMOs has manifested itself in calls for increased government regulation in the form of a ban on GMOs in the food supply or mandatory labelling. Government policymakers are faced with the challenge of balancing human safety concerns and other risks with the potential benefits offered by GMOs. They are increasingly under pressure from consumer groups to require labelling of the products of GMOs. Food manufacturers have also been pressured by consumer groups, and some companies have sought to promise consumers that their products are free of GMOs (Baker and Burnham, 2002).

In many European Countries, support is lukewarm. The majority of the population refuse food containing GMOs. There is a certain demand for clear and strict labelling of GMFs. Overall support for GMFs is seen in only four countries; Spain, Portugal, Ireland and Finland. These varying degrees of acceptance show that Europeans continue to distinguish between different types of applications, particularly medical in contrast to agri-food applications. There seems to be much less concern about the consumption of genetically modified foods in the USA than Europe (Moseley, 1999). Polls indicate that American consumers are largely unaware that they are already eating foods with ingredients from GM crops (Arntzen et al, 2003). Consumer acceptance studies of GM food products in Canada, Japan, Norway, the USA and the UK show that the majority of those surveyed want labelling for GM content, but in experimental and real-market tests in North America, the presence of GM-labelled food has not had a significant impact on actual purchase decisions (Smyth and Phillips, 2003).

In Turkey, consumers are largely unaware that they are already eating foods with ingredient from GM crops. A newspaper on GMFs was published and `*No to GMOs Platform*` against GM Foods have been established in order to

highlight the unclear effects of GM crops on environment, human and animal health (www.gdoyahayir.org, 21-August-2004). Additionally, articles about the

uncontrolled consumption of GM foods began to take place in some journals Rejections against to consumption of GM Foods without control mechanisms and necessity of related regulations were emphasised (Gıda Dergisi, 2004).

To respond the citizens expectations and demands, consumers should be informed based on the scientific data. Although public awareness toward genetic engineering is rapidly increasing, the low level of knowledge and the distrust in risk regulators is still contentious issue. Information providers on GM foods, should provide the development of an effective way of communicating risks and benefits to the public.

1.15. Objective of this study

The main objective of this study is to analyse and evaluate the raw and processed maize samples chosen arbitrarily from the Turkey food market. In Turkey, there is no detailed information about the presence and absence of genetically manipulated crops either consumed as food or feed. In order to set strict standards for the protection of human, animal health and environment, detection and quantification studies are indispensible. Labelling requirements should be implemented and consumers should be informed. This study cause the paying attention the situation of maize with respect to genetic modification placed in Turkey Food market pointing following steps:

First of all, maize samples both raw materials as maize kernels consumed by human and animals and processed foodstuff as maize flour, maize starch, maize chips, corn flakes, popcorn and baby food were randomly collected from different bazaars and supermarkets located in Turkey. DNA`s of 31 samples were extracted by CTAB isolation method manually and after determining the concentrations of DNA, theoretical detection limits were calculated.

Secondly, in order to check the presence and quality of DNA extracted from maize and maize derived foods, primer sets specific to zein gene found in all kind of maize were designed and PCR reaction was carried out.

Thirdly, in order to achieve the demonstration of presence or absence of genetic modification, screening methods were carried out by means of primer sets which are complementary to CaMV 35S promoter and NOS terminator, most frequently used promoter and terminator regions in commercially available genetically modified crops. The most sensitive detection method, Polymerase Chain Reaction, was used for screening purposes. Primers sets for kanamycin resistant genes were designed screening was enlarged by addition of detection of kanamycin resistant gene. Detected sequences by conventional PCR were verified by sequence analysis. Furthermore screening was also carried out via Real-Time PCR for NOS terminator and 35S promoter sequences.

Fourtly, so as to identify Bt11 GM maize lines primer set specific Bt11 gene construct was designed. Event specific PCR was carried out.

Lastly, to conduct quantification and to demonstrate the practical detection limits, Real-time PCR was used.

CHAPTER II

MATERIALS AND METHODS

2.1. Sampling

Samples utilised in this study were represented in Table 2.1. Analysed samples involve raw materials consumed as food and feedstuff and processed foods consisting of maize chips, breakfast cereal, pop corn, maize starch, and maize flour. Commercial samples of processed maize and raw maize were purchased from local supermarkets and bazaars located in Turkey. Serious attention was given for collecting different brands of each sample.

So as to detect %1 genetic modification level of sample, at least 299 grain which is almost equivalent to 50 g is indispensable. From representative 1 kg samples of grains not less than 300 grains were chosen at random and blended in a laboratory blender (Anthony laboratory blender) until the raw sample of maize were completely ground into fine powder. The homogenised maize grains were mixed throughly.

During blending, sterilised equipments were used. In order to avoid contamination towards the sampling, samples that did not contain any genetic modification as conventional non-modified maize lines (which were obtained from Ministry of Agriculture and Rural Affairs, Ankara Province Control Lab., Biogenetic Unit) were prepared first in a place that has never exposed to GMOs before. Samples were packaged and labelled after homogenisation procedure and stored at $+4^{o}C$ for further use.

Analysed samples were demonstrated in Table 2.1.

Table 2.1. Raw and processed maize samples

Sample number	Sort of maize sample	Origin	Location
1	Animal	ND	Eskişehir
2	feed	ND	Eskişehir
3		ND	Istanbul
4		USA	Eskişehir
5		USA	Ankara
6		Argentina	Ankara
7	*Food*	South Africa	Isparta
8		Argentina	Ankara
9		Argentina	Antalya
10		Argentina	Canakkale
11	Maize flour	ND	Ankara
12		ND	Ankara
13		ND	Istanbul
14		ND	Ankara
15		ND	Ankara
16	Maize starch	ND	Ankara
17		ND	Eskişehir
18		ND	Ankara
19		ND	Ankara
20	Corn flakes	ND	Eskişehir
21		ND	Ankara
22		ND	Istanbul
23	Maize chips	ND	Ankara
24		ND	Ankara
25		ND	Ankara
26	Pop corn	ND	Istanbul
27		ND	Ankara
28		ND	Ankara
29	Conventional non-	Turkey	Adapazarı
30	modified maize	Turkey	Adapazarı
31		Turkey	Adapazarı

ND: Non Determined

There will be a relation between the processing stage of samples and yield of DNA extraction/PCR amplification, thus processing stages of by-products have been taking into account during the study.

2.2. Certified Reference Materials (CRMs)

Certified Reference Materials (CRMs) produced by European Union (EU) Joint Research Center, Institue for Reference Materials and Measurements (IRMM) were purchased from Fluka. These were freeze dried homogenised powders of Bt-11 maize (IRMM-412R) with different mass fractions as 0.0%, 0.1%, 0.5%, 1%, 2%, 5 % (w/w). These material is part of a set of CRMs containing different mass fractions of maize powder prepared from genetically modified (GM) Bt-11 maize.

They are available in the form of glass bottles containing approximately 1 g of maize powder packed under argon atmosphere. CRMs were stored dry and in the dark at +4 °C. Table 2.2. demonstrates the CRMs.

Table 2.2. List of CRMs of Bt-11 maize

Certified Reference Materials (CRMs)	Product code
0% Bt11 (w/w)	IRMM 412R-0
0.1% Bt11 (w/w)	IRMM 412R-1
0.5% Bt11 (w/w)	IRMM 412R-2
1.0% Bt11 (w/w)	IRMM 412R-3
2.0% Bt11 (w/w)	IRMM 412R-4
5.0% Bt11 (w/w)	IRMM 412R-5

2.3. Chemicals

The chemicals and their suppliers were listed in Appendix A.

2.4. Buffers and Solutions

Composition and preparations of buffers and solutions are given in Appendix B.

2.5. Enzymes and Markers

Enzymes and markers which were used in this study are listed in Appendix C with their suppliers.

2.6. DNA extraction from raw and processed foods

DNA extraction from maize foods was performed by using method of Rogers & Bendich (1985). 100 mg of ground homogeneous sample was transferred into a sterile 1.5 ml reaction tube. 300 μl sterile dH_2O was added and mixed with a loop. Then 500 μl of CTAB-buffer (20g/l CTAB, 1,4 M NaCl, 20Mm EDTA, 100Mm Tris-HCl) was transferred to the reaction tube. 20 μl of Proteinase K (20mg/ml) was added and shaked gently. The mixture was incubated at 65 ^{o}C for 30-90 minutes in water bath. Before the last 5 minutes of incubation 20μl of DNAse free pancreatic RNAse (10mg/ml) was added and tubes were incubated at 65 ^{o}C for 5 minutes. Tubes were centrifuged in microcentrifuge Hettich Micro 12-24 for 10 minutes at 13.000 ×g. In order to remove significant quantities of protein, supernatant was carefully collected with a micropipet and transferred to a tube containing 500 μl chloroform/isoamylalcohol (in the ratio at 24/1 respectively). After a gently mix for 30 s, sample was centrifuged for 5 minutes at 13.000 rpm. The upper layer of separated liquid was collected and transferred to a new sterile tube. 2V of CTAB precipitation buffer (5 g/l CTAB, 0.04M NaCl) was added and the sample was mixed by pipetting. Then tube was kept at room temperature for 60 minutes. So as to obtain pellet, tubes were spinned for 5 minutes at 13.000 ×g. Supernatant was discarded and pellet was collected. After dissociation of precipitation in 350 μl NaCl (1.2 M), 350 μl of chloroform: isoamylalcohol (in the ratio at 24/1 respectively) was added and gently mixed for 30 s. Then tubes were recentrifuged for 10 minutes until phase separation occurs. The upper layer was transferred to a new reaction tube. 0.6 volume of isopropanol was added and gently mixed in order to precipitate nucleic

acid. Tubes were centrifuged for 10 minutes at 13.000 ×g. Supernatant was discarded and 500 μl of 70% ethanol solution was added. After a gentle mixing, tubes were centrifuged for 10 minutes at 13.000 ×g. Supernatant was discarded. Pellet was collected and samples were kept at room temperature

until the ethanol completely evaporated. Collected pellet was redissolved in 100 μl sterile dH_2O (Somma, 2002).

Nucleic acids were analysed by agarose gel electrophoresis. DNA of maize was kept at –20 °C for further use.

2.7. Determination of DNA concentration

The amount of DNA present in a sample was measured spectrophotometrically. The quality of isolated DNA was estimated by using a spectrophotometer. Calibration of spectrophotometer was performed and standard deviation from ideal line was shown by graph. After calibrating spectrophotometer and calculating standard deviation, blank was measured by sterile dH_2O. A sample of DNA was diluted with sterile dH_2O in the ratio of 1/10 and placed in a cuvette. Absorbance was measured at 260 nm and 280 nm. The ratio of A_{260}/A_{280} represents the purity of the sample (ratios of 1.8=pure, 2.0≥RNA contamination, 1.6≤ protein contamination). DNA concentrations were expressed as ng/μl.

Formula used for determination of DNA concentration was as below.

$$\text{DNA}_{\text{Concentration}} = OD_{260} \times 50 \times \text{Dilution factor}$$

OD_{260} stands for optical density in A_{260} and 50 represents the A_{260} unit of ds DNA (Somma, 2002)

2.8. Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal electrophoresis apparatus, BioRad Gel Electrophoresis System. For visualizing genomic DNA on agarose gel,

0.8 % agarose gel was prepared in 1X TAE Buffer (Appendix B). 1.5 μl EtBr from a stock solution of 15 μg/μl was added to 30 ml of melted gel at 50-60 °C. The gel was placed into the electrophoresis tank filled with 1X TAE buffer for covering the gel. Electrophoresis was performed at 125 mA and 80 volt for 60 minutes.

Finally, the gel was transferred to an UV transilluminator and DNA was viewed at 260 nm and photographed.

2.9. Primer design for Polymerase Chain Reaction (PCR)

Specific primers were designed according to foreign genes transformed to Genetically Modified (GM) crops. Primer 3 programme was used during primer design (http://www-genome.wi.mit.edu/cgi-bin/ primer/ primer3 , 10-11-2003). In order to find the specificity of primers to sequences BLAST and CLUSTALW were utilised.

2.9.1. Maize Specific Primers

The primers *Zein-3* and *Zein-4* (Querci and Mazzara, 2002) specific to maize zein gene (ze1, coding for 10 kb protein) was used to confirm the presence and quality of DNA extracted from raw maize material and processed maize foods. Expected product size was 277 bp (Table 2.3.).

Primer binding sites of zein specific primers and amplification regions of zein genes were shown in Appendix D.

2.9.2. Detection primers for GM maize

In order to determine regulatory sequences as Cauliflower 35S promoter derived from Cauliflower Mosaic Virus (CaMV35S) and Nopalin Synthase (NOS) terminator derived from *Agrobacterium tumefaciens*, specific primers were designed. Sequences of CaMV35S promoter and NOS terminator primers were obtained from Lipp and Broadmann (Lipp, et al, 1999).

In order to detect Kanamycin resistance gene in collected samples, primer pairs were designed. DNA sequences of the Kanamycin resistance genes (nptII) were obtained from GenBank databases (www.ncbi.nlm.nih.gov/Pubmed, 03-05-2003). The sequences, characteristics and binding sites of designed detection primers were illustrated in Table 2.3.

Sequence homology between primer sets and obtained sequence was performed via BLAST (http://www.ncbi.nlm.nih.gov/blast, 03-05-2003). Multiple sequence alignment conducted via Clustal W Homology Programme (www.expasy.org, 03-05-2003) was shown in Appendix D.

2.9.3. Cry specific primers

In order to perform specific detection of Bt11 maize lines which conveys CryIAb genes , event specific PCR was conducted by IVS2/PAT B primer sets giving a PCR product of 189 bp. These primer sets were specific to IVS2-PAT B border, instead of CryIAb gene, thus specific detection of Bt11 maize lines was achieved. Sequences of IVS2/PAT B primer pairs were obtained from Federal Institue for Health Protection of Consumers (BgVV), (BgVV, 1999).

Detailed information of IVS2/PAT B primer pairs were illustrated in Table 2.3.

All primer pairs were synthesised by IONTEK, Turkey.

Table 2.3. Primers utilized and designed for detection and identification

PRIMERS	TARGET SEQUENCE	ORIGIN OF SEQUENCE	PRIMER NAME	PRIMER LENGHT	*SEQUENCE*	PCR PRODUCT LENGHT	REFERENCES
Control primers	Zein	Zea mays	Ze –03 (reverse) Ze-04 (forward)	19 bp 21 bp	AgTgCgACCCATATTCCAg gACATTgTggCATCATCATTT	277 bp	Querci et al,2002
Detection primers	CaMV 35S promoter	*Agrobacterium tumefaciens*	35S-1 (reverse) 35S-2 (forward)	19 bp 20 bp	gCTCCTACAAATgCCATCA GATAgTgggATTgTgCgTCA	195 bp	Lipp, et al, 1999
	NOS terminator	*Agrobacterium tumefaciens*	NOS-01 (reverse) NOS-02 (forward)	20 bp 20 bp	gAATCCTgTTgCCggTCTTG TTATCCTAgTTTgCgCgCTA	180 bp	Lipp, et al, 1999
	Kanamycin	*E.coli* Tn5	Kan-01 (reverse) Kan-02 (forward)	18 bp 18 bp	gAAggCgATAgAAggCgA TTgCTCCTgCCgAgAAAg	453 bp	AF274974
	Cry1Ab (Event specific primer)	*Bacillus thuringiensis*	IVS2-2/PATB (forward) IVS2-2/PATB (reverse)	22 bp 22 bp	CTgggAggCCAAggTATCTAAT gCTgCTgTAgCTggCCTAATCT	189 bp	BgVV, 1999

2.10. Polymerase Chain Reaction

The Polymerase Chain reaction (PCR) was carried out on a Techne Progene and Techne 455 thermocycler machines. For each series, a master mix was prepared. Each PCR reaction mix contained 1X PCR Buffer (Fermentas), including $MgCl_2$ (Fermentas), dNTP (Fermentas), Taq Polymerase (Fermentas), and ddH_2O. Reactions were conducted with 5 μl of template DNA and 25 μl of reaction mix. In order to check out reagent contamination, one tube was prepared with ddH_2O instead of template DNA.

2.10.1. Plant Specific PCR

A Plant DNA check system via zein primer sets (*Zein-3* and *Zein 4*) was conducted. PCR assays were performed in the final volumes of 30 μl in 0.2 ml tubes containing 1X reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5μM of each zein primers, 0.025 U/μl of Taq Polymerase in the Techne Progene. 2 μl of DNA template was used in the amplification reaction. The final volume of 30 μl was completed by sterile double distilled water. Ingredients of a 30 μl reaction mixture was illustrated in the Table 2.4.

Table 2.4. PCR reaction mixture for plant specific PCR

Reaction mixture	Final concentration
Sterile dd H_2O	-
25 Mm $MgCl_2$	2.5 mM
10X PCR Buffer	1X
10Mm dNTP mix	0.2 mM
Taq DNA Polymerase	0.025 U/μl
Reverse Primer	0.5 μM
Forward Primers	0.5 μM
DNA	5 ng

As positive control, pure DNA isolated from conventional non-modified maize sample was used. As negative control, pure DNA isolated from tomato and soybean , which didn't convey the zein gene was used. 0.5 ml eppendorf

tubes were incubated in a Techne Progene thermocycler under the following programme (Table 2.5.).

Table 2.5. PCR conditions for plant specific PCR

PCR programme	Cycles	Temperature	Time
Initial denaturation		95 °C	3 min
Denaturation	35 cycle	96 °C	1 min
Annealing		60 °C	1 min
Extension	-	72 °C	3 min

The PCR products were analysed using 1% agarose gel in BioRad electrophoresis system. A screening system was then conducted for the samples giving positive results via zein specific primer sets.

2.10.2. Screening of Genetically Modified Maize via PCR

A screening system, Polymerase Chain Reaction (PCR), was applied to identify the 35S promoter, NOS terminator and Kanamycin resistance genes (Kan^R). PCR assays were performed in the final volumes of 30 μl in 0.2 ml tubes containing 1X reaction buffer, 1.5 mM $MgCl_2$ for CaMV and Kan^R genes and 3 mM $MgCl_2$ for NOS genes, 0.2 mM dNTP, 0.5μM of each primers, 0.025 U/μl of Taq Polymerase in the Techne Thermocycler machine. 2 μl of DNA template was used in the amplification reaction.

PCR conditions for amplification of NOS terminator and CaMV 35S promoter were as in the Table 2.6.

Table 2.6. PCR Conditions for CaMV 35S promoter and NOS terminator

PCR programme	Cycles	Temperature	Time
Initial denaturation		95 °C	3 min
Denaturation	35 cycle	96 °C	1 min
Annealing		60 °C	1 min
Extension	-	72 °C	3 min

The final volume was completed to 30 μl by adding sterile double distilled water. Ingredients of 30μl reaction mixture for screening purpose was demonstrated in the Table 2.7.

Table 2.7. Ingredients of PCR reaction mix for screening primers.

Reaction mixture	Final concentration
Sterile dd H_2O	-
25 Mm $MgCl_2$	1.5 mM(35S and Kan^R) 3 mM(Nos)
10X PCR Buffer	1X
10Mm dNTP mix	0.2 mM
Taq DNA Polymerase	0.025 U/μl
Reverse Primer	0.5 μM
Forward Primer	0.5 μM
DNA	10 ng

PCR conditions for detection of Kan^R was demonstrated in Table 2.8.

Table 2.8. PCR conditions for Kanamycin resistance (Kan^R) gene

PCR programme	Cycles	Temperature	Time
Initial denaturation		94 °C	2 min
Denaturation	30 cycle	94 °C	40 sec
Annealing		57 °C	1 min
Extension	-	72 °C	10 min

2.10.3. Identification of insect resistant maize via PCR

PCR assay was performed in the final volume of 30 μl in 0.2 ml eppendorf tubes containing 1X reaction buffer, 3 mM $MgCl_2$, 0.2 mM of dNTP mix, and 0.5 mM of each primer, 1.5 units of Taq DNA polymerase. 5 μl of template DNA was used in the amplification reactions (Table 2.9.)

Table 2.9. Ingredients of PCR master mix for IVS2/PAT primer pairs.

Reaction mixture	Final concentration
Sterile dd H_2O	-
25 Mm $MgCl_2$	3 mM (for Bt11 specific)
10X PCR Buffer	1X
10Mm dNTP mix	0.2 mM
Taq DNA Polymerase	0.025 U/μl
Reverse Primer	0.5μM
Forward Primer	0.5μM
DNA	5 ng

Samples were subjected to PCR with the following temperature profiles with IVS2/PAT B primer pairs (Table 2.10).

Table 2.10. PCR conditions for IVS2/PAT B primer pairs.

PCR programme	Cycles	Temperature	Time
Initial denaturation		94 °C	2 min
Denaturation	40 cycle	94 °C	45 sec
Annealing		56 °C	1 min
Extension		72 °C	1 min
Final Extension	-	72 °C	10 min

Amplification products were separated by 1% agarose gel made visible by Ethidium Bromide (EtBr) and transferred to UV transilluminator for photographing.

2.11. Sequencing of specific genes

Detected kanamycin and Bt11 genes was confirmed by sequencing. Verification of specific genes was conducted via automatic sequencing. Specific genes were sequenced by IONTEK. The sequencing results were both compared with the target sequences that were used for primer design and

were analysed with the BLAST programme. On the other hand CaMV 35S promoter and NOS terminator sequences were verified via Real Time PCR.

2.12. Real Time PCR

2.12.1. Screening of maize samples by Real Time PCR

In order to carry out screening of maize samples, Real time PCR was conducted by LightCycler GMO screening Kit in Roche LightCycler instrument. The solutions of GMO screening kit were briefly centrifuged in Hettich Micro 12-24 rotor. In order to protect from light, tubes were wrapped with folio. The master mixes for the 35S promoter/NOS terminator and Plant PCR must be separately set-up, using the respective detection mixtures. 11 μl ddH_2O, 2 μl 35S/NOS detection mix (10X concentration), and 2 μl LightCycler GMO Screening enzyme were transferred to an 1.5 ml eppendorf tube. For plant gene detection, another mixture was prepared by adding 11 μl ddH_2O, 2 μl plant gene detection mix (10X concentration), and 2 μl LightCycler GMO Screening enzyme into the 1.5 ml reaction tube.

The tubes containing reaction mix was gently mixed and 15 μl of the reaction mix was transferred to the LightCycler Capillary. 5 μl template DNA was added to the capillary. For the negative control, 5 μl PCR grade H_2O was added. For the positive control 5 μl LightCycler GMO screening control was added. The adapters containing capillaries were placed into the microcentrifuge and spinned briefly before transferring to instrument. Following conditions were applied for quantitative screening of 35S/NOS genetic elements (Table 2.11). The given data was recorded.

Table 2.11. Conditions for screening by Real Time PCR

PCR programme	Cycles	Temperature	Time
Pre incubation	1 cycle	95 °C	15 sec
Denaturation		95 °C	15 sec
Annealing	45 cycle	60 °C	25 sec
Extension		72 °C	15 sec
Final Extension	1 cycle	40 °C	30 sec

2.12.2. Quantification by Real-Time PCR

For quantitative detection of Bt11 maize line, Real-time PCR was carried out by Sure Food-GMO Bt11 Corn Kit in Cepheid-SmartCycler. The components of Sure Food-GMO Bt11 Corn Kit was spinned shortly in a microcentrifuge Hettich Micro 12-24 for 10 seconds at 13.000 ×g in order to collect the reagents in the tube. Since tube components should be kept in dark, all tubes was wrapped with folio. In order to plot standard curve, at least 3 reactions are needed for *zein* and *ivs*. One no template control and two positive control should be performed for each. Two reaction mix was prepared. For each reaction of the zein detection(corn reference gene) 17 μl Zein –reaction mix was mixed with 1 μl FDE and 0.1 μl Taq- Polymerase in to 1.5 ml eppendorf tube. Reagents are mixed well by pipetting.

For each reaction of the ivs-detection (GMO detection gene) mix 17 μl of ivs-reaction mix, 1.0 μl FDE and 0.1 μl of Taq-Polymerase were mixed in 1.5 ml eppendorf tube. Due to the pipetting loss of reagents, 10% zein-LC and ivs-LC mix were prepared more than actually needed.

Standard DNA was diluted in 1:10 steps in TE buffer in order to prepare different DNA concentrations for the standard curves of the reference gene (*zein*) and the detection gene (*ivs*). Standard DNA provided by the kit contains 10^6 copies of both targets (*zein* and *ivs*). The copy numbers of the dilutions was shown in Table 2.12.

Table 2.12. Dilution levels of the standard DNA

Dilution level	1	2	3	4	5
Zein	$1x10^5$	$1x10^4$	$1x10^3$	$1x10^2$	$1x10^1$
ivs	$1x10^5$	$1x10^4$	$1x10^3$	$1x10^2$	$1x10^1$

For SmartCycler Real-Time machine, special eppendorfs provided from SmartCycler were used. 18 μl of zein-LC reaction mix was transferred to SmartCycler eppendorfs. In order to avoid contamination caps of eppendorfs closed immediately. Template DNA was not added into the first eppendorf

and it was used as no template control in order to check the contamination. 2 μl of standard DNA from tube 1 which contains $1x10^5$ copy DNA, 2 μl other standard DNA from tube 3 which contains $1x10^3$ copy DNA, another 2 μl of standard DNA from tube 5 containing $1x10^1$ was transferred into following 3 eppendorfs. 2 μl of the sample DNA was added in to the next capillaries. The cap of the capillaries were closed immediately. The same procedure was repeated for the detection gene (*ivs*). All SmartCycler eppendors were centrifuged in SmartCycler Rotor for 10 seconds.

The channel 1:20 was adjusted after measuring the fluorescence level in the real time fluorimeter option. The following profile was set up for Real-Time quantification (Table 2.13).

Table 2.13. SmartCycler setup for quantification of ivs.

PCR programme	Cycles	Temperature	Time
Initial denaturation		95 °C	1 min
Denaturation	45 cycle	95 °C	5 sec
Annealing		62 °C	10 sec
Extension		65 °C	15 sec
Final Extension	-	40 °C	10 sec

Positive control should contain 2% GMO. If this number is not met a correction factor K can be calculated. By the formula of

K=GMO content reference gene DNA/calculated number

2.13. Affect of heat treatment on DNA isolation and PCR amplification

Maize kernels were boiled for 5 min, 15 min, 30 min, 1 hour, 1,5 hour, 2 hour and 3 hour. DNA extraction by CTAB method (Rogers and Bendich, 1985) was applied. DNA isolation was followed by plant specific PCR. The results were visualised by agarose gel electrophoresis.

CHAPTER III

RESULTS AND DISCUSSION

3.1. DNA extraction

DNAs of collected samples were extracted by using the method of Rogers & Bendich (1985). Extracted DNA was subjected to agarose gel electrophoresis with DNA size marker, *Lambda DNA* digested with *PstI* restriction endonuclease (fragment sizes: 11501, 5077, 4749, 4507, 2838, 2556, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264). High molecular weight DNA was extracted from maize kernels. Even though processed foods were exposed to heat and pH treatment, maize DNAs were also isolated from them. By gel electrophoresis, quality of DNA can be defined by fragment length. If the high molecular weight genomic DNA is present, it can be described as intact. Isolated DNAs from processed food were not always intact and fragment molecular weights of extracted DNA varies between 100 bp to 10.000 bp. During processing of foods, exposure to heat, low pH and nucleases result in hydrolysis, depurination and enzymatic degradation. Thus, intact DNA can not be extracted from processed foods. Infact, as it is well established that heat treatment of foods results in significant reduction in the fragment wholeness of genomic DNA (Gachet et al, 1999). DNA isolated from processed foods is usually of low quality and available target sequences may be rather short (Hemmer, 1997). Since the processed foods are deep fried, heated and filtered, very low amount of DNA was extracted. It seems that poping, crushing, filtering either destroys DNA by applied pressure or applied heat (Pauli et al, 2000). Foods which were only concentrated, cut, heated, crushed or popped were considered as low processed. Foods which were precipitated, deep fried roasted or steamed were considered as medium processed and foods which were fermented or extruded were considered as highly processed (Pauli et al, 2000).

Upon these definitions, except for corn flakes and maize chips, all chosen samples could be described as low processed.

Among the analysed foods, the less processed sample was represented by maize flour. In terms of processing stages, maize flour was followed by pop corn, maize starch, maize chips and corn flakes. Yield of DNA extraction from maize flour, was higher than yield of DNA extraction from other processed foods.

Processing stages of these by-products derived from maize was illustrated in Table 3.1. Processing levels were demonstrated as plus signals. The more plus indicates, the more processing steps. The flowcharts of processed foods were shown in Appendix F.

Table 3.1. Processing stages of maize foods (Pauli et al, 2000).

Sort of the sample	Processing stage
Maize kernels	+
Maize flour	++
Popped popcorn	+++
Maize starch	++++
Maize chips	+++++
Cornflakes	++++++

3.2. Determination of DNA concentration and Theoretical Detection Limit

According to results of spectrophotometer measurement, DNA concentrations were calculated. As an expected result, the concentration of the extracted DNA from raw materials were higher than the concentration of processed material. Isolations were repeated, providing that the extracted DNA from the same food matrices were not approximately in the same concentration and quality. After calculating DNA concentration, theoretical detection limits and copy numbers were estimated. The DNA amount in the unreplicated haploid nuclear genome of an organism is referred to as its C value. Based on the 1C value, 100 ng maize DNA contains 3.8×10^4 copies of maize genome (Bonfini et al, 2001). By

this ratio, theoretical detection limits and copy numbers were calculated for each DNA. The results were demonstrated in Table 3.2.

Table 3.2. Concentrations and theoretical detection limits of extracted DNA

SAMPLE SORT	NO	CONCENTRATION	COPY NUMBER	THEORETICAL DETECTION LIMITS
Feed	1	11.85 ng/µl	4503	0.022%
	2	11.20 ng/µl	4256	0.023%
	3	10.15 ng/µl	3857	0.025%
	4	8.35 ng/µl	3173	0.031%
	5	9.09 ng/µl	3454	0.028%
Food	6	9.25 ng/µl	3515	0.028%
	7	10.6 ng/µl	4028	0.024%
	8	10.1 ng/µl	3838	0.026%
	9	8.00 ng/µl	3040	0.032%
	10	11.05 ng/µl	4199	0.023%
Maize flour	11	6.40 ng/µl	2432	0.041%
	12	9.40 ng/µl	3572	0.028%
	13	7.70 ng/µl	2926	0.034%
	14	6.20 ng/µl	2356	0.042%
	15	5.05 ng/µl	1919	0.052%
Maize starch	16	5.10 ng/µl	1938	0.051%
	17	5.00 ng/µl	1900	0.050%
	18	8.80 ng/µl	3344	0.030%
	19	5.90 ng/µl	2242	0.044%
Corn Flakes	20	4.22ng/µl	1603	0.062%
	21	4.47 ng/µl	1698	0.058%
	22	5.65 ng/µl	2147	0.046%
Maize Chips	23	4.32 ng/µl	1643	0.068%
	24	4.82 ng/µl	1831	0.054%
	25	5.72 ng/µl	2173	0.046%
Pop Corn	26	5.05 ng/µl	1919	0.052%
	27	5.02 ng/µl	1907	0.052%
	28	7.22 ng/µl	2743	0.036%
Conventional non-modified maize	29	10.00 ng/µl	3857	0.025%
	30	13.05 ng/µl	4951	0.020%
	31	13.45 ng/µl	5111	0.019%
Certified Reference Materials	32	13.00 ng/µl	4940	0.020%
	33	12.65 ng/µl	4807	0.020%
	34	15.36 ng/µl	5836	0.017%
	35	13.05 ng/µl	4959	0.020%
	36	14.02 ng/µl	5327	0.018%
	37	12.80 ng/µl	4864	0.020%

By means of genome copy numbers, GM copy numbers was calculated for Certified Reference Materials (CRMs). GM copy numbers of CRMs can be observed in Table 3.3.

Table 3.3. GM copy numbers of CRMs

CRMs	Genome Copy number	GM copy number
% 0 Bt11 CRMs	4940	0
% 0.1 Bt11 CRMs	4807	4.807
% 0.5 Bt11 CRMs	5836	29.18
% 1.0 Bt11 CRMs	4959	49.59
% 2.0 Bt11 CRMs	5827	106.54
% 5.0 Bt11 CRMs	4864	243.2

3.3. Plant specific PCR

All processed food samples were checked out by maize specific primer sets, ze-03 and ze-04, yielding a 277 bp PCR product (Table 2.3). During development of foods, various ingredients are mixed and subjected to different processes. In addition to adverse affect of heat and pH treatment, ingredients exist in the food matrix can be isolated with DNA and result in impeding of PCR reaction. Thus, applying plant specific PCR before GMO detection is unavoidable. A maize specific PCR with the primer pair specific to zein gene was performed. 277 bp PCR products were generated from all samples. The results indicated that the isolated target maize DNAs were amplifiable and in a well quality for PCR reactions.

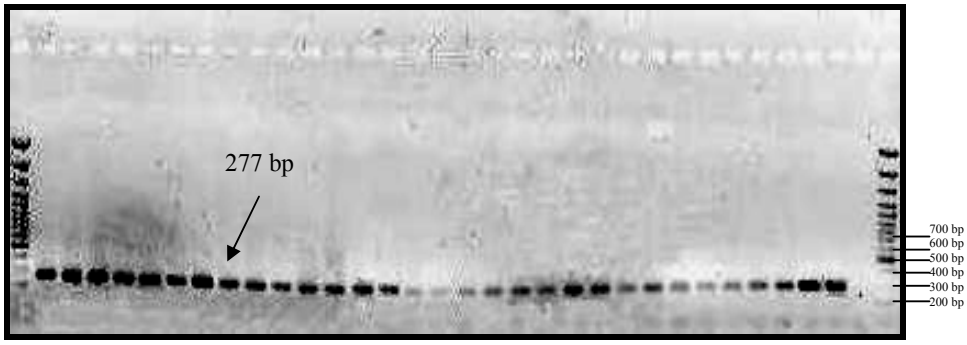
As can be seen in the Figure 3.1., amplification by all raw materials yielded almost equal concentration of PCR products while amplification by processed materials gave approximately same intensity of PCR product but their brightness were less than those of the raw materials. Table 3.4. illustrates the lanes for agarose gel of zein specific PCR.

Table 3.4. Lanes of plant specific PCR

Figure 3.1-A			Figure 3.1-B	
Lanes	Sample sort	Sample id	Lanes	Sample Sort
Lane 1-5	Maize kernels (Feed)	1-5	Lane 1	No template
Lane 6-10	Maize kernels (Food)	6-10	Lane 2	Tomato DNA
Lane 11-15	Maize flour	11-15	Lane 3	Soybean DNA
Lane 16-19	Maize starch	16-19	Lane 4	0% CRMs
Lane 20-22	Corn flakes	20-23	Lane 5	0.1% CRMs
Lane 23-25	Maize chips	24-26	Lane 6	0.5% CRMs
Lane 26-28	Pop corn	27-29	Lane 7	1% CRMs
Lane 29-31	Non-modified kernel	30-31	Lane8	2% CRMs
Lane 32	No template	-	Lane 9	5% CRMs

(A)

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 M



(B)

M 1 2 3 4 5 6 7 8 9 M

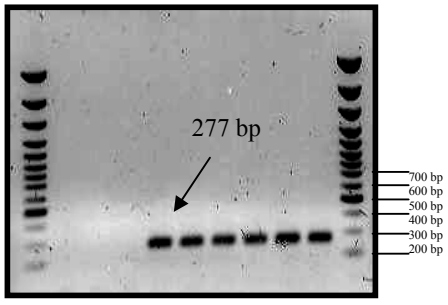


Figure 3.1. Visualised Plant specific PCR .A lane 1-10 PCR products of raw materials; lane 11-28 PCR products of processed samples; lane 29-31 conventional non-modified maize seeds originating from Turkey; lane 32 no-template **B** Lane 1 no template DNA; lane2 amplification with tomato DNA; lane 3 amplification with soybean DNA; lane 4-9 amplification with CRMs

These results pointed out that; our extracted DNA from food material contains maize DNA. No template DNA which is used for the control of PCR contamination didn't give an amplicon. Soybean and tomato DNAs were utilised to control the specificity of ze-03 and ze-04 primer pairs. No positive signals were obtained by soybean and tomato DNA as well. Binding sites of primer pairs and the sequence of amplified PCR product produced by Ze-03 and Ze-04 were illustrated in Appendix D.

3.4. Screening PCR

In order to check presence and absence of transgenes as promoter, terminator and antibiotic resistance genes, screening was conducted. Screening only displays the presence of transgene and it should be pursued by an identification PCR or an event specific PCR.

3.4.1. Screening for CaMV35S promoter

To investigate the presence of CaMV35S, the primer pair 35S-1 and 35S-2 (Table 2.3.) resulting in 195 bp of PCR product was used for PCR amplications.

Table 3.5. Lanes of Screening PCR for 35S promoter

Figure 3.2			
Lanes	Sample sort	Lanes	Sample Sort
Lane 1	No template	Lane 8-12	Maize kernels (Feed) Sample id, 1-5
Lane 2	5% CRMs	Lane 13-17	Maize kernels (Food) Sample id, 6-10
Lane 3	0% CRMs	Lane 18-22	Maize flour Sample id 11-15
Lane 4	0.1% CRMs	Lane 23-26	Maize starch Sample id, 16-19
Lane 5	0.5% CRMs	Lane 27-29	Corn flakes Sample id, 20-22
Lane 6	1% CRMs	Lane 30-32	Maize chips Sample id, 23-25
Lane 7	2% CRMs	Lane 34-35	Pop corn Sample id, 26-28
		Lane 36-37	Non-modified kernel Sample id, 29-30

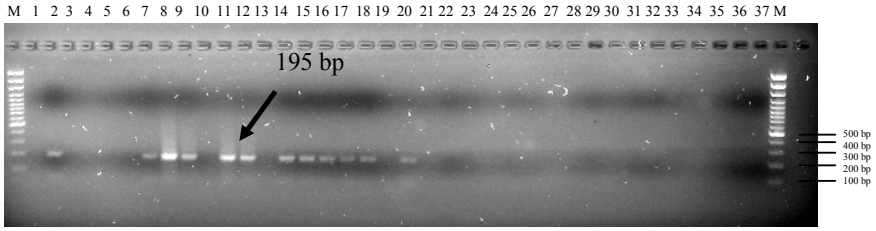


Figure 3.2. PCR amplification by 35S-1 and 35S-2 primer sets

100 bp DNA ladder was used for comparision. As negative control, 0% Bt11 and conventional non-modified maize lines originating from Turkey (provided by Ministry of Agricultural and Rural Affairs, Ankara Province Control Lab, Biogenetic Unit) were used. Utilized positive controls were 0.1%, 0,5%, 1%, 2%, 5% CRMs of Bt11 maize lines. It can be observed in the Figure 3.2. that , no amplification was detected neither with the negative controls, nor with the PCR contamination control (no template DNA). Detection of CaMV35S promoter in raw material was observed by conventional PCR whereas no detection was observed in processed material except for maize flour which is the lowest processed food among the analysed samples.

Limit of detection (LOD) by 35S-1 and 35S-2 primer sets was clarified as 2%. Positive amplification signal was only generated by 2% and 5% CRMs. No positive signals were provided for 0.1%, 0.5% and 1% CRMs. It can be concluded that their GM DNA amount was below the LOD.

In some transgenic maize lines, enhanced duplicated 35S promoter was used. This sequence carries a second complementary annealing site for the 35S-1 primer. Therefore, 457 bp fragment together with a 195 bp fragment were detected from e-P-35S (Jaccaud et al, 2003). When 30 cycle was applied for PCR, apart from 195 bp of fragment, 457 bp of fragment was observed for some unknown samples in this study.

There exist some handicaps for detection of processed maize samples. Firstly maize content of foods is relatively be diminished according to the processing stage. Secondly, ingredients of processed food matrixes may be isolated with DNA during extraction, thus they might affect the PCR. As a last possibility, the processed foods giving a negative amplification signal may be developed from conventional non-modified maize lines and they are completely unmodified.

3.4.2. Screening for Nos terminator sequence

In addition to CaMV 35S promoter, the most commonly used genetic element taking a crucial role in the gene expression is Nos terminator. Primer sets of NOS-01 and NOS-02 were used so as to obtain 180 bp amplicon.

As negative controls, 0% Bt11 and conventional non-modified maize lines were used. As positive controls, 0.1%, 0,5%, 1%, 2%, 5% CRMs of Bt11 maize line were used. No detection was found in 0.1%, 0.5% and 1% Bt11 maize lines. On the other hand, 180 bp amplicon was obtained in 2% and 5% Bt11 maize lines. It can be concluded that, in these conditions, detection limit was 2% by NOS-01 and NOS-02 primer sets.

Figure 3.3. represents the PCR amplifications by NOS-01 and NOS-02 primer pair. Remarkably, except for maize flour no detection was observed in processed maize lines. The maize flour is the food, exposed to less process. Therefore detection could be possible for maize flour. The processed maize samples may face with limit of detection like 0.1%, 0.5% and 1% Bt11 CRMs.

Table 3.6. Lanes of Screening PCR for NOS terminator

Figure 3.3			
Lanes	Sample sort	Lanes	Sample Sort
Lane 1	No template	Lane 8-12	Maize kernels (Feed)
Lane 2	5% CRMs	Lane 13-17	Maize kernels (Food)
Lane 3	0% CRMs	Lane 18-22	Maize flour
Lane 4	0.1% CRMs	Lane 23-26	Maize starch
Lane 5	0.5% CRMs	Lane 27-29	Corn flakes
Lane 6	1% CRMs	Lane 30-32	Maize chips
Lane 7	2% CRMs	Lane 34-35	Pop corn
		Lane 36-38	Non-modified kernel

Lanes of agarose gel electrophoresis for Nos screening was shown in Table 3.6.

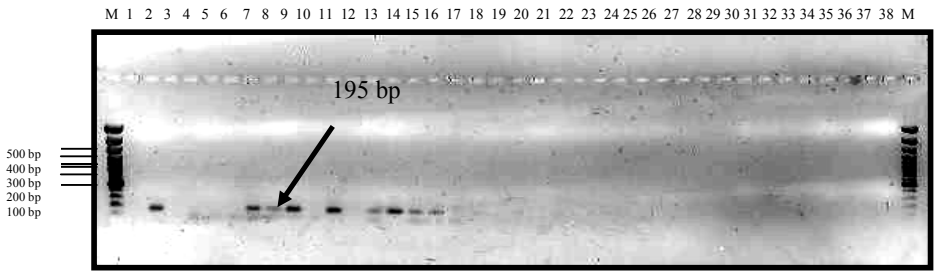


Figure 3.3. PCR amplification of NOS terminator sequences

3.4.3. Screening for Kanamycin resistance (Kan^R) sequences

Expression of Kanamycin resistance gene confers resistant to kanamycin and it is used as a marker gene in the construction of transgenic plants, allowing the selection of transformed cells (Jaccaud et al, *2003*). Among commercialised maize lines , Mon series maize lines convey Kanamycin resistance gene. In Bt11 maize line, ampicillin resistance genes was used but after transformation, the antibiotic resistance gene was removed. Therefore, detecting ampicillin resistance gene is not possible in Bt11 maize lines.

Finding kanamycin resistance gene implies the presence of Mon series maize lines.

In order to display Kanamycin resistance gene, PCR was carried out by Kan-01 and Kan-02 primer sets generating a 453 bp amplicon (Table 2.3.). Mon series of transgenic maize lines conveys kanamycin resistance gene. 5 % Mon810 CRMs was used as a positive control. As a negative control, conventional non-modified maize lines and 5% Bt11 maize line were used. No detection was observed neither in conventional non-modified maize lines nor in 5% Bt11 maize line. Three maize samples whose sample numbers are 2, 7 and 13 (with lane number 3, 5 and 10) gave a positive amplification signal. It can be concluded that these samples were member of Mon series maizes and conveys manipulated DNA.

Table 3.7. Lanes of Screening PCR for Kan^R

Figure 3.4			
Lanes	Sample sort	Lanes	Sample Sort
Lane 1	No template	Lane 14-18	Maize flour Sample id,11-15
Lane 2	5%Bt 11	Lane 19-22	Maize starch Sample id, 16-19
Lane 3	5%Mon 810	Lane 23-25	Corn flakes Sample id,20-22
Lane 4-8	Maize kernels (Feed) Sample id, 1-5	Lane 26-28	Maize chips Sample id, 23-25
Lane 9-13	Maize kernel (Food) Sample id, 6-10	Lane 29-31	Pop corn Sample id, 26-28
		Lane 32-33	Non-modified kernel Sample id, 29-30

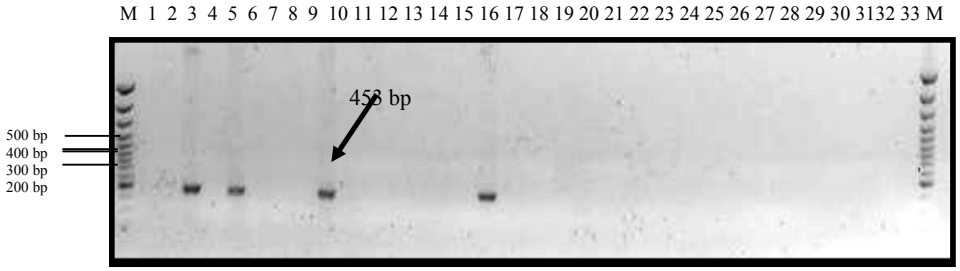


Figure 3.4. PCR amplification via Kan ^{R} primer sets

3.5. Screening via RT-PCR

In addition to conventional PCR, screening was conducted via LightCycler Real-Time PCR System. During the RT-PCR assay the target gene was amplified, simultaneously recognised and monitored by fluorescent probe. The light emitted from the dye was received by a computer and shown on a graph. PCR cycles was displayed on the X axis and logarithmic indications of fluorescent intensity was shown on the Y axis. For each sample Ct values of the specific target genes (CaMV 35S promoter and Nos terminator) and reference gene (plant specific gene) were determined. Furthermore, plant specific gene was also checked for each sample. Since the hybridisation probes were used in addition to primer sets, no verification experiment was necessary after RT-PCR assay. In all

graphs, PCT stands for positive control of target gene and NCT represents negative control of target gene. Moreover, PCR indicates the positive control of reference genes and NCR means negative control of reference genes. Colours of fluorescent signals for each sample were displayed near the graph. % 0.1 CRM was used in order to find detection limit. The general results were located in Table 3.8.

3.5.1. Screening of NOS terminator via RT-PCR

In order to detect NOS terminator via RT-PCR assay, 3 runs were conducted. The total fluorescence signals for each run was in Appendix G. In each run, positive and negative controls were used not only for target gene (NOS terminator gene) but also for reference gene (plant specific gene). The positive controls were provided by the kit. As a negative control ddH_2O was used instead of DNA.

To clearly demonstrate the fluorescence signals, one negative, one positive fluorescence signals were picked from software in addition to fluorescence signals of negative and positive controls. Fluorescent signals of positive and negative controls were represented in the Figure 3.5. Positive controls gave fluorescence signals which were above the threshold level whereas fluorescent signals of negative controls couldn't pass the threshold line. It indicates that our results were free of contamination and reliable.

The Figure 3.6 illustrates the fluorescent signals of one unknown samples in addition to fluorescence signals of positive and negative controls. It can be concluded that the unknown sample was non-GM, since it couldn't pass the threshold level.

In Figure 3.7, an unknown sample giving a positive result was illustrated in addition to fluorescence signals of positive and negative signals. The target gene of our unknown sample was represented by dark green and reference gene for our unknown sample was illustrated by brown colours.

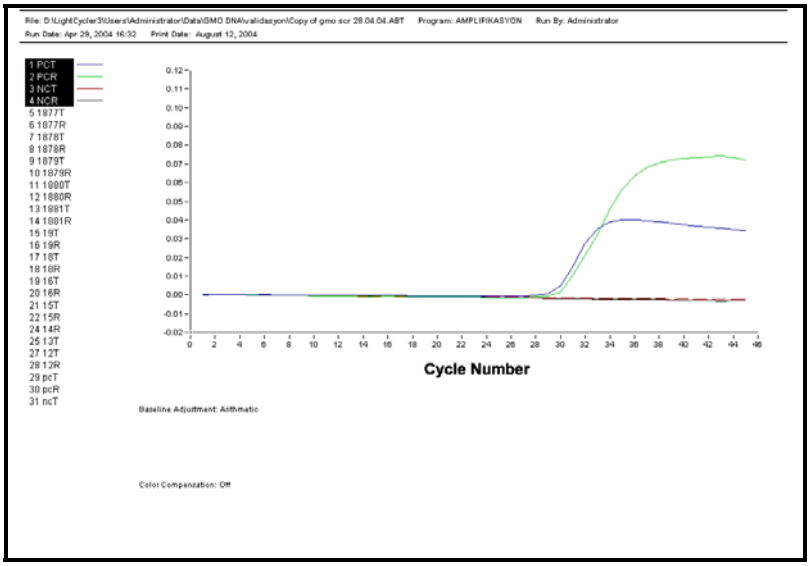


Figure 3.5. Fluorescent signals of positive and negative controls for t-nos

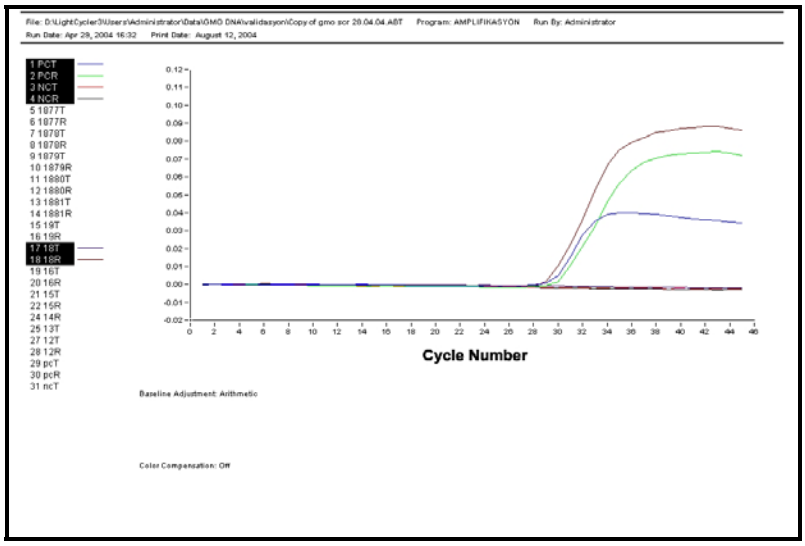


Figure 3.6. Negative signal of unknown sample for t-nos.

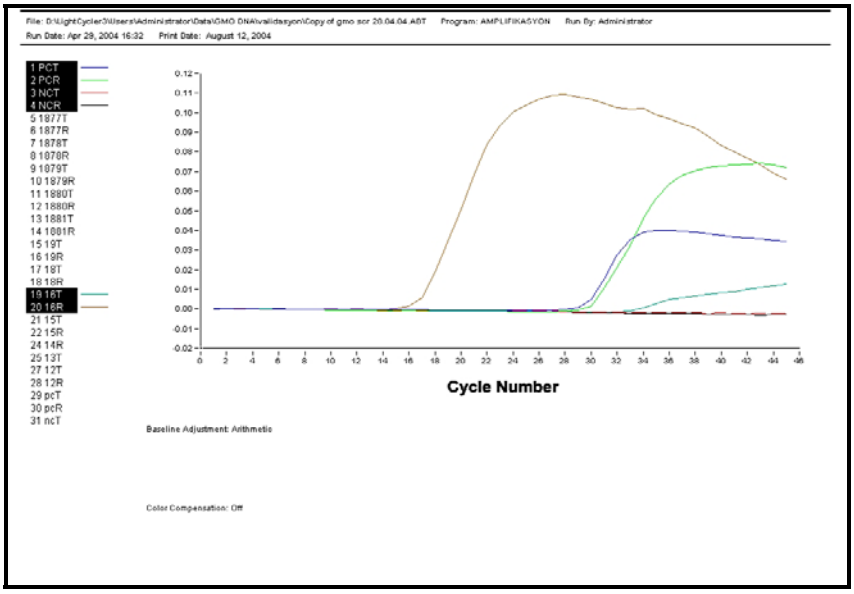


Figure 3.7. Positive signal of unknown sample for t-nos.

General results was shown in Table 3.8. Total fluorescence signals were represented in Appendix G.

3.5.2. Screening for CaMV 35S promoter via RT-PCR

In addition to NOS terminator sequences, CaMV 35S promoter was also detected via Real-Time PCR system. To clearly demonstrate fluorescence signals, 3 graphs were picked from the software. These are; unknown sample giving negative result, unknown sample giving a positive result and positive and negative controls. Figure 3.8. represents the fluorescence signals of negative and positive controls. Blue and green lines, above the threshold level, illustrates the positive controls of target and reference genes. Red and black lines below the threshold level displays the negative controls of target and reference genes. As an expected result, no positive signal was obtained from negative controls. Figure 3.9 illustrates the unknown sample giving a negative result. Dark green line represents the reference gene of unknown sample while pink line shows the target gene of unknown sample. Since the fluorescent signal of target gene below the threshold level, it was accepted as non-GM.

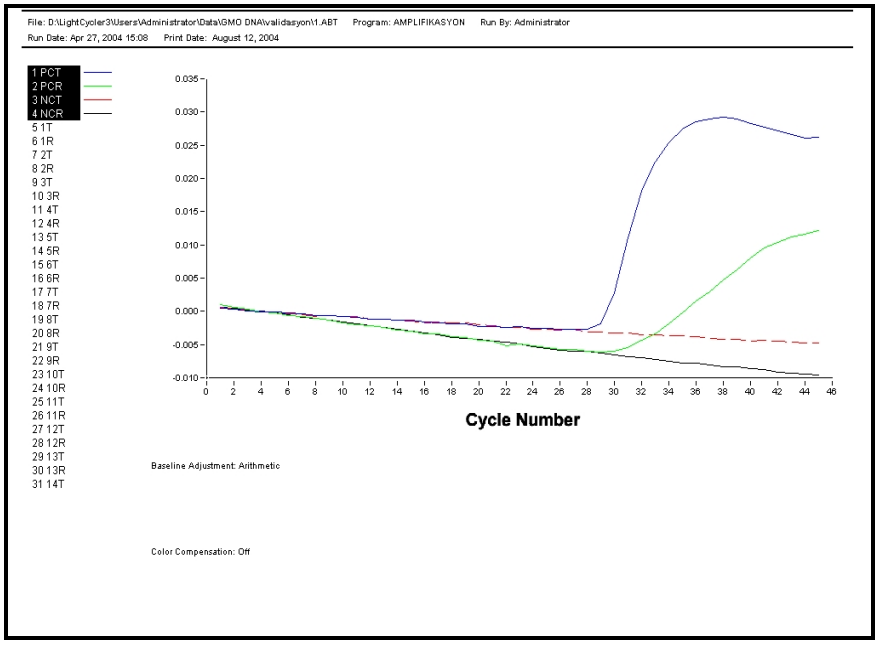


Figure 3.8. Negative and positive controls for CaMV 35S

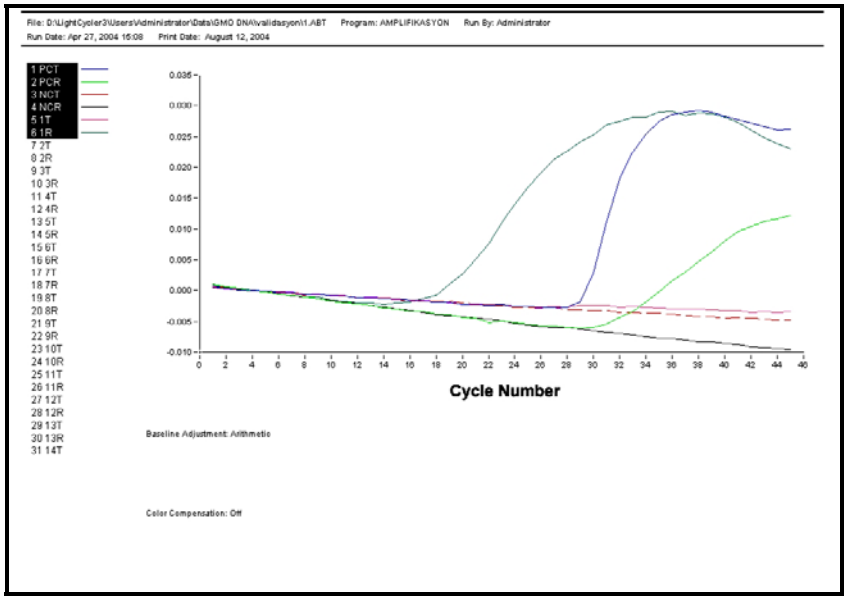


Figure 3.9. Negative signal of unknown sample for CaMV 35S

One unknown sample giving a positive result can be seen in the Figure 3.10. The fluorescence signals of reference and target gene could pass the threshold level. Therefore, it was accepted as GM maize.

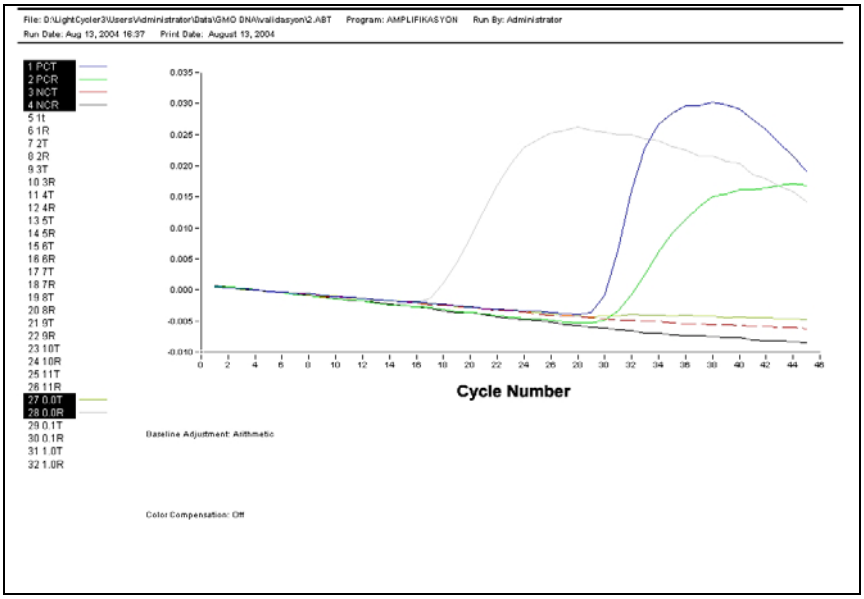


Figure 3.10. Positive signal of unknown sample for CaMV 35S

Total fluorescence signals were displayed in Appendix G.

0.1% CRMs was used in order to determine detection limit. 0.1% Bt11 gave the positive signal for both NOS terminator and CaMV 35S promoter. This result indicates that practical detection limit was 0.1% via RT-PCR whereas it was 2% via conventional PCR. Thus it could be emphasised that RT-PCR was more sensitive and reliable than traditional PCR. There was a crucial difference between limit of detection of RT-PCR and conventional PCR.

Table 3.8. demonstrates the screening results for NOS terminator, CaMV 35S promoter and plant specific gene.

Table 3.8. General results of samples for screening via RT-PCR

Sample	Zein	CaMV	Nos
1	+	+	-
2	+	+	-
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	-
7	+	+	-
8	+	+	+
9	+	+	+
10	+	+	-
11	+	+	+
12	+	+	-
13	-	+	-
14	+	-	-
15	+	-	-
16	+	+	+
17	+	-	-
18	+	-	-
19	-	-	-
20	-	-	-
21	+	-	-
22	+	-	-
23	+	-	-
24	+	-	-
25	+	-	-
26	+	-	-
27	+	-	-
28	+	-	-
29	+	-	-
30	+	-	-

In Table 3.8., sample numbers are according to Table 2.1. Plus sign indicates the presence of transgene whereas minus sign shows the absence of transgene.

3.6.Event specific PCR

Bt 11 transgenic corns involves CryIAb gene which is under the control of CaMV 35S promoter and Nos terminator. Thus, samples giving a positive amplification signals for NOS terminator and 35S sequences were selected for event specific PCR. Primer pairs of IVS2/PATB was used which is solely specific to Bt11 maize lines (Table 2.3). The fragment of 189 bp was observed after gel electrophoresis. The PCR products were run with size marker, 100 bp DNA

ladder. As a positive control 0.1%, 0.5%, 1%, 2% and 5% Bt11 CRMs were used. Among the Bt11 CRMs, detection was observed only in 2% and 5% Bt11 maize lines which means that detection limit by these primer sets was 2%. 0.1 %, 0.5% and 1% Bt11 maize lines faced with limit of detection. No template reaction mixture for the control of PCR contamination and 0% Bt11 CRMs didn't give any visible band. Eight maize samples whose sample numbers are 1, 6, 8, 9, 10 and 12 (with lane number 7, 12, 14, 15, 16 and 18) gave a positive amplification signal. Samples The result of gel electrohoresis was shown in Figure 3.11. and described in Table 3.9.

Table 3.9. Lanes of event specific PCR

Figure 3.11			
Lanes	Sample sort	Lanes	Sample Sort
Lane 1	5% CRMs	Lane 7-11	Maize kernels (Feed) Sample id, 1-5
Lane 2	0% CRMs	Lane 12-16	Maize kernel(Food) Sample id, 6-10
Lane 3	0.1% CRMs	Lane 17-21	Maize flour Sample id, 11-15
Lane 4	0.5% CRMs	Lane 22	Non-modified kernel Sample id, 29-30
Lane 5	1% CRMs	Lane 23	No template
Lane 6	2% CRMs		

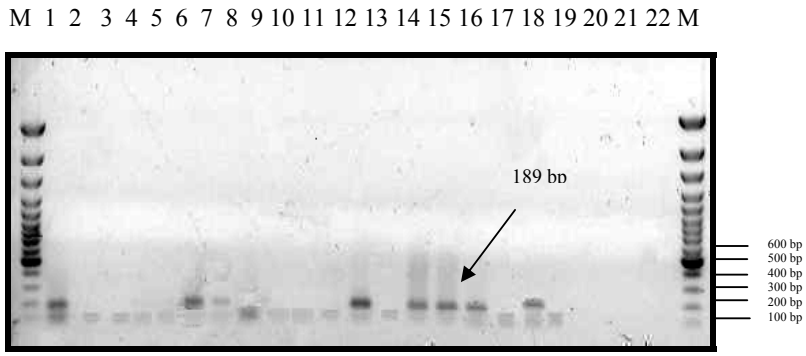


Figure 3.11. PCR amplification for Bt11 maize lines.

3.7. Impact of processing

During processing, the frequently used such techniques as crushing, milling, extruding or filtering accelerates the DNA degradation. Continuously heat treatment degrades DNA and strongly reduce average fragment length. The degredation of DNA by heat treatment was emphasised in some studies (Meyer *et. al.*, 1994).

So as to point out impact of heat treatment into detection, the maize kernels were boiled for 5 minutes, 10 minutes, 30 minutes, 1 hour, 1.5 hour, 2 hours and 3 hours. As a sample, maize kernel was chosen which gave positive signal via all primer sets. DNA extracted from unboiled maize was used as control. CTAB method was used for DNA extraction.

For gel analysis Lambda DNA digested with PstI was used as marker. As can be seen in Figure 3.12., after 10 minute boiling, there was a significant reduction in isolated DNA amount. DNAs isolated from boiled maize after 30 minutes didn't show any high molecular weight DNA. Slightly weaker band was visualised in 30 min boiled DNA. More than 1 hour boiled samples, degraded DNA pieces were observed on EtBr stained agarose gel. High molecular weight DNA can only be isolated from 5 min and 15 min and 30 min boiled DNA. DNA isolated from maize kernels boiled for 1 hour to 3 hour completely destroyed and gave the fragment of 100 bp. Lanes and samples were given in Table 3.10.

Table 3.10. Impact of heat treatment on DNA isolation

Lanes	Boiling time
Lane 1	Unboiled DNA
Lane 2	5 min
Lane 3	15 min
Lane 4	30 min
Lane 5	1 hour
Lane 6	1.5 hour
Lane 7	2 hour
Lane 8	3 hour

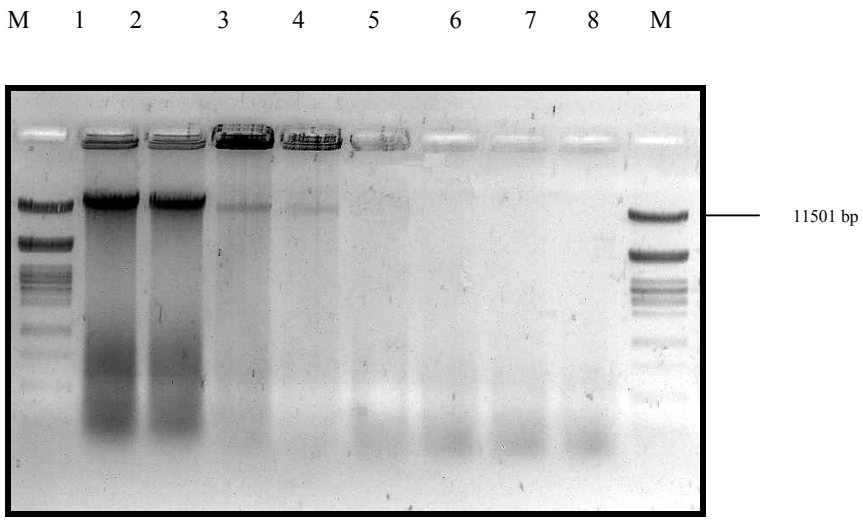


Figure 3.12. Impact of heat treatment on DNA isolation.

DNA isolation was pursued by plant specific PCR. In order to observe effect of heat treatment on amplification , samples were subjected to PCR via zein specific primers. Optimised temperature and time profile which were described in the previous chapter were utilised. 100 bp DNA size marker was used for size comparision. Owing to DNA degradation, zein genes were amplified only in 5 minutes, 10 minutes and 30 minutes boiled maize kernels. 277 bp of slightly week zein band was obtained in DNA which was extracted from 1 hour boiled maize. No detection was observed in 1.5 hour, 2 hour boiled sample (Figure 3.13. and Table 3.11.)

Table 3.11. Impact of heat treatment on PCR amplification

Lanes	Boiling time
Lane 1	Unboiled DNA
Lane 2	5 min
Lane 3	15 min
Lane 4	30 min
Lane 5	1 hour
Lane 6	1.5 hour
Lane 7	2 hour
Lane 8	No template

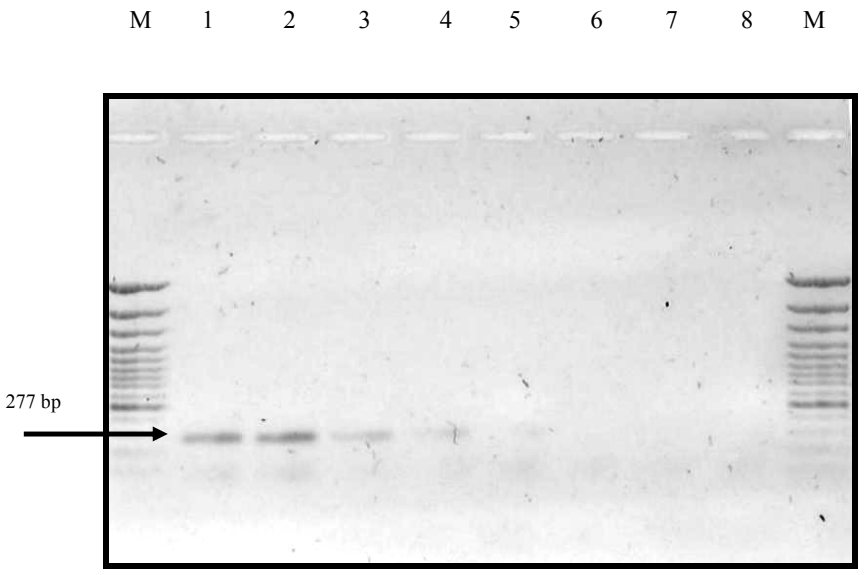


Figure 3.13. Impact of heat treatment on PCR amplification.

Undetectable results of transgenes in processed maize samples can be explained by reduction in the DNA content and copy number. Opposite to transgene detection, maize specific genes can be amplified via zein specific primer sets.

Failure in detecting GM can be explained as follows. Low content of genetically modified maize in the food affects the detection. Furthermore substances present in the food matrices may affect the PCR by either interfering with the target DNA or inhibiting the enzymatic reactions. Finally physical and chemical parameters such as shear forces, heat treatment and nuclease activities may lead to degredation of DNA (Straub et al, 1999).

Hupfer and his co-workers demonstrated that the detection of Bt maize by PCR in polenta is strongly dependent on the pH during thermal treatment of product. They also couldn't detect transgenes after 100 days ensilage by the primers yielding 1914 bp amplicon whereas they manage to detect maize specific gene and Bt specific gene via primer sets yielding 226 bp and 211 bp respectively (Hupfer et al, 1999). Therefore for the GM detection in processed foods, primer sets giving the shortest amplicons should be utilized.

DNA degrading factors as thermal treatment and restrictive pH values may diminish the content of amplifiable target DNA. It is impossible to simulate in one simple model all the factors which may affect DNA in food during processing together with ingredients that may have inhibitory effects. This could only be realised by adding target DNA to various food matrixes subject to food processing. However this approach is not realistic bearing in mind that, for example, more than 20,000 soybean products with different matrices are on the market (Jankiewicz et al,1999).

Analysis of maize products seems to be more difficult, probably due to the large genome size of maize and subsequently the lower number of gene copies extracted from the matrix (Lipp et al, 1999).

Kay and Van den Eede pointed that, there is serious implications for the practibility of GMO detection in food. The amount of unreplicated haploid genome present in a sample is useful for relating genome copy number to the amount of sample taken. For example up to 36, 697 copies of haploid *Zea mays* genome are present in a typical 100 ng DNA analytical sample given the 1C. It follows that the single copy of haploid maize genome in a 100 ng DNA present at a level of 0.0027%. Levels of DNA below this threshold simply cannot be detected reliably in samples. A second problem is sampling errors This occurs in a perfectly homogenous preparation even if a large amount of DNA is extracted from the laboratory sample. As the amount of DNA extracted from the sample becomes lower, sampling errors becomes larger (Kay, and Van den Eede, 2001).

In this study, among the processed foods, detection was accomplished only in maize flour which is the lowest processed material. Detection limit was directly related to isolated maize DNA with high quality. When DNA of maize flour was subjected to agarose gel electrophoresis , it was demonstrated by high molecular weight.

3.8. Quantification via RT-PCR

In this study, quantitative detection was conducted with SmartCycler Real Time system. The real time PCR system result in the detection of PCR amplification during the early phases (logarithmic phase) of reaction. Thus, it creates crucial advantage over conventional PCR detection. In contrast to end-point determinations, RT-PCR systems monitor the reaction as it actually occurs in real time. Therefore, it is faster and easier than conventional PCR.

Threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the logarithmic phase of the amplification for the most accurate reading. Cycle threshold (Ct) is the value at which sample crosses threshold level. These two values are very crucial in evaluating the data analysis.

In order to detect and quantify the unknown maize samples, standard curves were plotted not only for GM target gene (Bt11) but also for reference gene (plant specific gene). Standard DNA which was obtained from the kit was seriously diluted and used as a template to plot a standard curve. Obtained standard curves were shown in Figure 3.14. A linear relationship between log input DNA and Cycle threshold (Ct) values was displayed. For housekeeping gene and for target gene two different standard curves were plotted. Two runs were performed and for each run same standard curves were used. Blue points represents the standard curve points obtained by diluted standard DNA. The red point located on standard curve represents the positive control. Each standard curve has one equation which demonstrates its own slope. Coefficients were calculated by the software.

The equation for the standard curve of ivs was shown below,

$$y=-0.35X+12.357$$

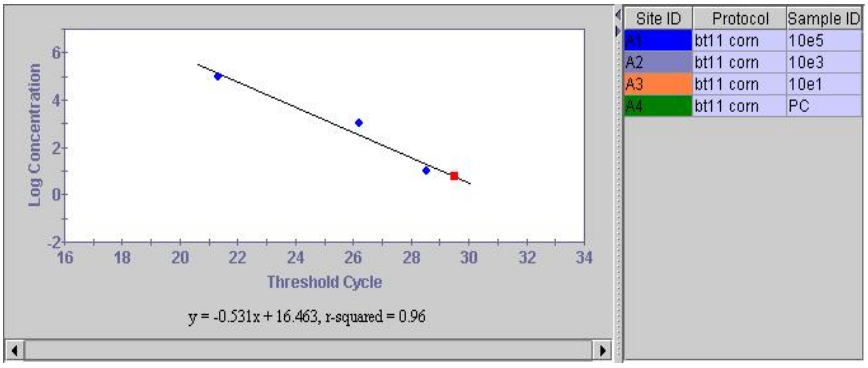
The equation for the standard curve of zein was given below,

$$y=-0.531X+16.463$$

-0.35 and –0.531 indicates the slopes of standard lines. 12.357 and 16.463 was calculated by software and mathematical modelling of slopes were created.

In Appendix H, the location of sample 4, 5, 6, 7 was demonstrated on standard curve as an example.

(A)



(B)

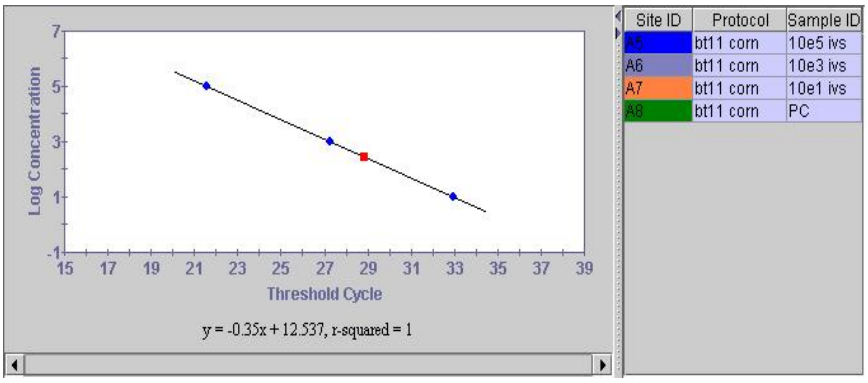


Figure 3.14. A. Standard curve of plant specific gene , Ct value versus log concentration of DNA **B.** Standard curve of GM target gene, Ct value versus log concentration of DNA

PC stands for positive control in Figure 3.14. R^2 represents the standard deviation. For zein and ivs , R^2 was 0.96 and 1 respectively.

Table 3.12 demonstrates the Ct values and copy numbers of diluted samples for plotting standard curve.

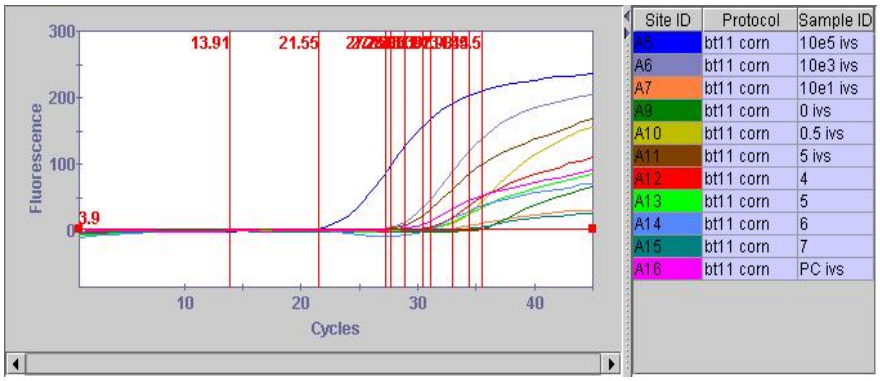
Table 3.12. Ct values and copy numbers of diluted target DNA

Ivs Standard Curve		**Zein Standard Curve**	
y=-0.35x+12.537		y=-0.531x+16.463	
Copy #	Ct	Copy #	Ct
100000	21,55	100000	21,31
1000	27,25	1000	26,21
10	32,98	10	28,53

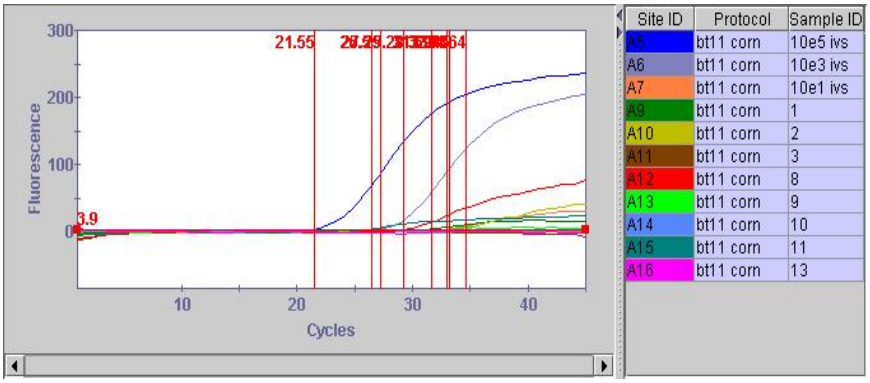
The fluorescence data analysis was performed by software. For each sample amount of the specific target gene and reference gene were determined by interpolation with the standard curve. Then GM content in another words genetic density was calculated as the ratio between GM target/reference gene amount. The % GM content was calculated by the formula below.

% GM content = GM target/ Reference gene x100

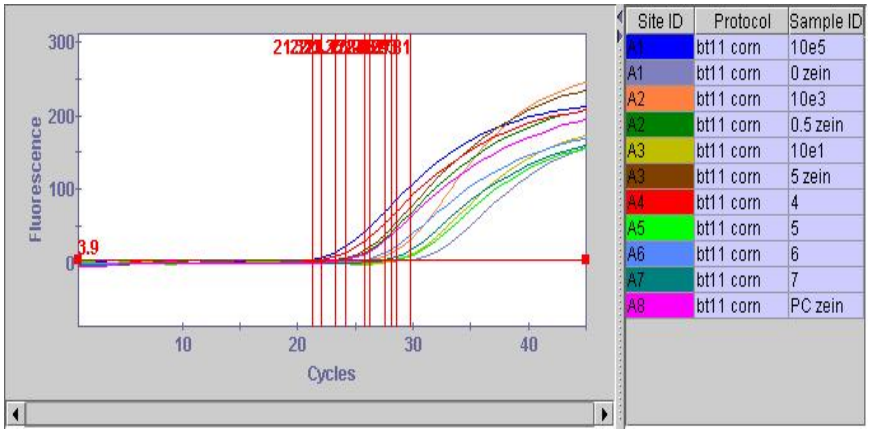
Thus, housekeeping gene amount and GM target gene amount were calculated in either case. For testing the reliablility of system CRMs were used. Profiles of two PCR runs and fluorescence signals given by software illustrated in Figure 3.15.


13.91
21.55
3.9
Fluorescence
Cycles
Site ID Protocol Sample ID
A5 bt11 corn 10e5 ivs
A6 bt11 corn 10e3 ivs
A7 bt11 corn 10e1 ivs
A9 bt11 corn 0 ivs
A10 bt11 corn 0.5 ivs
A11 bt11 corn 5 ivs
A12 bt11 corn 4
A13 bt11 corn 5
A14 bt11 corn 6
A15 bt11 corn 7
A16 bt11 corn PC ivs

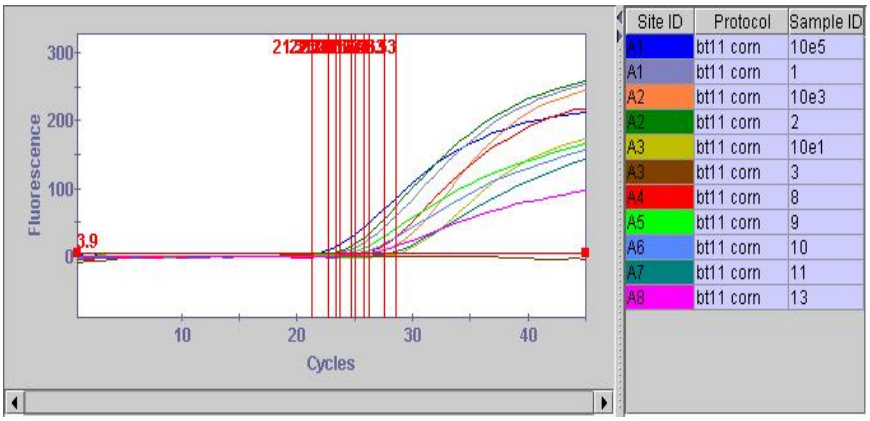
(A)


21.55
3.9
Fluorescence
Cycles
Site ID Protocol Sample ID
A5 bt11 corn 10e5 ivs
A6 bt11 corn 10e3 ivs
A7 bt11 corn 10e1 ivs
A9 bt11 corn 1
A10 bt11 corn 2
A11 bt11 corn 3
A12 bt11 corn 8
A13 bt11 corn 9
A14 bt11 corn 10
A15 bt11 corn 11
A16 bt11 corn 13

(B)


3.9
Fluorescence
Cycles
Site ID Protocol Sample ID
A1 bt11 corn 10e5
A1 bt11 corn 0 zein
A2 bt11 corn 10e3
A2 bt11 corn 0.5 zein
A3 bt11 corn 10e1
A3 bt11 corn 5 zein
A4 bt11 corn 4
A5 bt11 corn 5
A6 bt11 corn 6
A7 bt11 corn 7
A8 bt11 corn PC zein

(C)



(D)

Figure 3.15. Fluorescence signals fot Bt11 and zein genes **A.** First run Fluorescence signals for Bt11 **B.** Second run Fluorescence signals for Bt11 **C.** First run Fluorescence signal for plant specific gene **D.** Second run fluorescence signal for plant specific gene

The calculated results can be seen in Table 3.13. Ratio of GM target gene to zein gene gave the percentage of genetic modification content in unknown samples. K stands for correction factor. Calculated GMO content of the sample was corrected with this factor. The detection via RT-PCR was only applied to raw materials (sample 1-10) and two maize flour (sample 11 and 13).

Table 3.13. Summary of PCR results for RT-PCR quantification.

CALCULATED					
Samples	ivs	zein	ivs/zein	%	K
PC	277,608	4248,237	0,0653466	6,535	0,3060601
0% bt11	ND	4,282			
0.5% bt11	45,425	4478,567	0,0101428	1,014	0,3104292
5.0% bt11	679,06	12251,899	0,0554249	5,542	1,6963345
1	1838,524	61,729	29,783797	2978,380	911,56327
2	2,59	26445,703	9,794E-05	0,010	0,0029974
3	ND	ND			

Table 3.13 continued

Samples	ivs	zein	ivs/zein	%	K
4	ND	572,265			
5	80,295	52954,261	0,0015163	0,152	0,0464082
6	46,525	31,765	1,4646624	146,466	44,827475
7	8,703	7759,416	0,0011216	0,112	0,0343279
8	196,092	1431,348	0,1369981	13,700	4,1929665
9	3,04	61,962	0,0490623	4,906	1,5016023
10	28,085	11093,468	0,0025317	0,253	0,0774843
11	ND	2299,338			
13	ND	601,897			

As an expected result, ivs gene couldn't be detected in % 0 CRMs. In 0.5 % and 5 % Bt11 CRMs, without K factor (correction factor), read values were % 1.014 and % 5.542 respectively. With K factor, calculated values were % 0.31 and %1.69, for % 0.5 and %5 CRMs, respectively.

In sample 4, 11 and 13, ivs couldn't be detected. It can be concluded that they are not Bt11. In sample 3, neither plant specific gene nor ivs were detected by RT-PCR. The result indicated that RT-PCR should be repeated for sample 3.

Values obtained for sample 1 and 6, were above the standard curve points, Therefore, it should be emphasised that values for these two samples might be far from real quantification values.

The results of sample 1, 2, 3, 4, 6, 8, 9, 10, 11 and 13 were parallel to traditional PCR whereas the results of sample 5 and 7 were not parallel to traditional PCR results. It can be explained by limit of detection (LOD) of traditional PCR. For traditional PCR, limit of detection was 2%. In another words, for these samples, the amount of PCR products produced by conventional PCR were below the limit of detection.

By traditional PCR, limit of detection was 2% while by real time PCR it was 0.1%. Even the unknown samples contain the manipulated DNA, they may not be detected due to the detection limit. Thus, it can be emphasised that Real time PCR provides tremendous advantages for GMO detection.

Drawbacks of conventional PCR can be explained as follows. They are labour intensive, non-automated, and time consuming. Furthermore results of conventional PCR are based on size discrimination on agarose gel which may not be so reliable. EtBr staining is not so sensitive and quantitative. Additionally it is highly mutagenic . It is hard to differentiate between five fold change on agarose gel. RT-PCR is able to detect a two fold change. Real time PCR compensates for some detection errors caused by the conventional PCR. Real time PCR does not entail laborious post PCR methods. Therefore it brings about more faster and reliable results.

3.9. Verification of conventional PCR

Amplicons obtained by Kan ^{R} primer pairs were verified by sequence analysis. Since some amplicons as NOS terminator and CaMV 35S promoter were too short, they couldn't be verified by sequencing. Therefore Real-time PCR was conducted for verification purposes.

Homology between the sequence of PCR product and sequence obtained from gene bank was given in Figure 3.16 and 3.17.

- **Kanamycin resistance genes**

Multiple sequence alignment was carried out by CLUSTAL W (1.82). Alignment score is %94. Obtained sequence was compared with AF274974 which was collected from gene bank (http://www.ncbi.nlm.nih.gov/, 03-05-2003).

```
SEQUENCE        --------TNNTCNTGGGCTGATGCAATGCGGCGGCTGCATACGCTTNCCTCCGGCTACC 52
AF274974        GAAAGTATCCATCATGG-CTGATGCAATGCGGCGGCTGCATACGCTTGA-TCCGGCTACC 598
                         ** *** ****************************   **********

SEQUENCE        TGCCCATTCGACCACCNAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCC 112
AF274974        TGCCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCC 658
                ******* ******** ******************************************

SEQUENCE        GGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTG 172
AF274974        GGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTG 718
                ************************************************************

SEQUENCE        TTCGCCAGGCTCAAGGCGAGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGAT 232
AF274974        TTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGAT 778
                ***************** ******************************************
```

Figure 3.16. continued

```
SEQUENCE        GCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGC 292
AF274974        GCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGC 838
                ************************************************************

SEQUENCE        CGGCTGGGTGTGGCGGACCGCTATCAGGACATACACGTTGGCTACCCGTGATATTGCTGA 352
AF274974        CGGCTGGGTGTGGCGGACCGCTATCAGGACATA-GCGTTGGCTACCCGTGATATTGCTGA 897
                ********************************  ************************

SEQUENCE        AGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCNA 412
AF274974        AGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGA 957
                ************************************************************ *

SEQUENCE        TTCACAGCNCATCGNCTTCTATCNTCTTCATTTNNGNNT------- 451
AF274974        TTCGCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 1003
                *** ****  **** ********  **** *       *
```

Figure 3.16. Sequence result of Kanamycin resistance gene

❖ Ivs6/pat border

IVS2/PAT border is a junction region of Bt11 maize line. It was compared by two different sequence. Accession numbers of sequences obtained from gene bank was in Figure 3.17.

Multiple sequence alignment was carried out by CLUSTAL W (1.82). The first part of the obtained sequence compared with adh sequence. The second part of was aligned with PAT sequences.

```
HOMOLOGY BETEEN IVS2 AND SEQUENCE

AF050457 CCGACGTCTACTTCTGGGAGGCCAAGGTATCTAATCAGCCATCCCATTTGTGATCTTTGT 740
X04050   CCGACGTCTACTTCTGGGAGGCCAAGGTATCTAATCAGCCATCCCATTTGTGATCTTTGT 1934
M32984   CCGACGTCGACTTCTGGGAGGCCAAGGTATCTAATCAGCCATCCCATTTGTGATCTTTGT 1955
X04049   CCGACGTCTACTTCTGGGAGGCCAAGGTATCTAATCAGCCATCCCATTTGTGATCTTTGT 1940
SEQUENCE ------------------------------------CGCNTCCATTTGTGATCTTTGT 22
                                                **********

AF050457 CAGTAGATATGATACAACAACTCGCGGTTGACTTGCGCCTTCTTGGCGGCTTATCTGTCT 800
X04050   CAGTAGATATGATACAACAACTCGCGGTTGACTTGGCCCTTCTTGGCGGCTTATCTGTCT 1994
M32984   CAGTAGATATGATACAACAACTCGCGGTTGACTTGCGCCTTCTTGGCGGCTTATCTGTCT 2015
X04049   CAGTAGATATGATACAACAACTCGCGGTTGACTTGCGCCTTCTTGGCGGCTTATCTGTCT 2000
SEQUENCE CAGTAGATATGATACAACAACTCGCGGTTGACTTGCGCCTTCTTGGCGGCTTATCTGTCT 82
         ************************************************************

AF050457 TAGGGGCAGACTCCCGTGTTCCCTCGGATCTTTGGCCACGAGGCTGGAGGGTATGTTCTA 860
X04050   TAGGGGCAGACTCCCGTGTTCCCTCGGATCTTTGGCCACGAGGCTGGAGGGTATGTTCTA 2054
M32984   TAGGGGCAGACTCCCGTGTTCCCTCGGATCTTTGGCCATGAGGCTGGAGGGTATGTTCTA 2075
X04049   CAGGGGCAGACTCCCGTGTTCCCTCGGATCTTTGGCCATGAGGCTGGAGGGTATGTTCTA 2060
SEQUENCE CAGGGGCAGACTCCCGTGTTCCCTCGGATCTC--GACATGTCTCCGGAGAGGAGA--CCA 138
          ******************************  **** *** * **** *
```

(A)

```
HOMOLOGY BETWEEN PAT AND IVS2 SEQUENCE

AY562536 GCCGGCCATGTCTCCGGAGAGGAGACCAGTTGAGATTAGGCCAGC-TACAGCAGCTGATA 7278
AY562539 GCCGGCCATGTCTCCGGAGAGGAGACCAGTTGAGATTAGGCCAGC-TACAGCAGCTGATA 6779
AY562534 GCCGGCCATGTCTCCGGAGAGGAGACCAGTTGAGATTAGGCCAGC-TACAGCAGCTGATA 7278
SEQUENCE TCTCGACATGTCTCCGGAGAGGAGACCAGTTGAGATTAGGCNGGCCTACAGCAGCAGATT  170
                ********************************** *** *********  ***
```

(B)

Figure 3.17. Sequence result of ivs2/pat border

3.10. Interpretation of the result

Visualised bands for plant specific gene were generated by zein primer sets in all samples. As can be concluded from the plant specific PCR, the extracted target DNAs from food matrices were amplifiable maize DNA.

Most of the of commercialised GM crops contains either CaMV35S promoter or NOS terminator (BATS Report, 2003). It must be highlighted that while developing genetically manipulated plants, the 35S promoter and NOS terminator sequences were generally used together.

According to Table 3.14., in sample 1 and 2, 35S promoter was detected not only by RT-PCR but also via conventional PCR. In the same samples, Nos terminator couldn't be detected via RT-PCR whereas it was detected via conventional PCR. There may be a handling error for RT-PCR. In sample 1, Bt11 was detected by RT-PCR and by conventional PCR. In sample 2, no amplification was observed for Bt11 by conventional and RT-PCR. Kan ^{R} primer sets gave positive signal for sample 2. It indicates that, sample two might be a member of MON series maize lines.

In sample 3, no detection was obtained via conventional PCR for 35S promoter and Nos terminator. By RT-PCR, detection was observed for 35S promoter and Nos terminator. It indicates, the DNA amount of sample 3 is below the limit of detection (2%) for conventional PCR.

In sample 4, all the screening results were positive by conventional and real time PCR for 35S and NOS genetic elements. Neither in RT-PCR nor in conventional PCR, positive signal obtained for Bt11 maize line. In sample 5, for 35S promoter positive results were obtained not only by traditional PCR but also by RT-PCR. In sample 5, detection was possible only by RT-PCR for NOS terminator. Bt11 DNA was also found only by RT-PCR.

In sample 6, 35S promoter was solely detected by RT-PCR. For NOS terminator, detection was possible only by conventional PCR. Bt11 DNA was detected via RT-PCR and conventional PCR. In sample 7, we could detect 35S promoter by either RT-PCR or conventional PCR. Nos terminator couldn't be detected in sample 7 via RT-PCR . In sample 6 and 7, failure for detecting NOS terminator can be explained by handling error. In sample 7, Kanamycin resistance gene was detected as well. In sample 7 Kanamycin resistance gene was detected as well. Sample 10 may be a combination of Bt11 and MON series maize line. The maize kernels may be mixed during transportation and storage.

In Sample 8 and 9, except for Kanamycin resistance gene, all transgenes were detected. It means they were Bt11 maize line.

In sample 10, promoter sequence was detected not only by traditional PCR but also by RT-PCR. No detection was observed for NOS terminator. Sometimes it doesn't possible to detect NOS terminator sequences since NOS terminator might be spontaneously remove from gene cassette. In some plasmids as PV-ZMBK07, nos 3 truncation signal was lost through a 3 truncation of the hence cassette and therefore was not integrated into the genome (Querci et al, 2002). Raw materials were represented from sample 1 to 10.

Maize flours were numbered from sample 11 to sample 15. For sample 11, detection was possible for 35S promoter via Real-Time PCR. On the other hand NOS terminator was detected only by Real-Time PCR. Kanamycin resistance gene and Bt11 DNA were not detected by Real Time PCR and conventional PCR. In sample 12, detection of promoter region was only possible by RT-PCR. Bt11 maize DNA was detected by conventional PCR. No test was conducted for Bt11 detection via RT-PCR system. In sample 13, just 35S promoter was detected via RT-PCR. Neither Nos terminator, nor Bt11 DNA were found in sample 13. In

addition to CaMV 35S promoter , Kanamycin resistance gene was detected. In sample 14 and 15, negative signals were obtained for each transgene. Thus, sample 14 and 15 were completely non-GM.

Maize starch was represented from sample 16 to sample 19. For sample 16, 35S promoter and nos terminator detection was solely possible via RT-PCR which is more sensitive than other techniques It can be concluded that transgenes in maize starch is also detectable as maize flour. Applied treatments during processing of maize starch such as filtering, seperating, grinding do not affect detection. As maize flour processing , maize starch processing does not convey frying and very high heat treatment step. It may allow the extraction and detection of DNA . In sample 17 and 18, no detection was observed except for zein gene. It indicates that the sample 17 and 18 conveys non modified DNA. During the PCR, real time capillaries were broken for sample 19 and 20. Therefore zein gene couldn't be detected via RT-PCR system. The positive results were obtained via conventional PCR.

Sample 20, 21 and 22 represents the corn flakes while sample 23, 24 and 25 represents the maize chips. Moreover, sample 26, 27 and 28 represents the pop corn. No transgenes were found in corn flakes, maize chips and pop corn. Only zein genes gave positive signal via RT-PCR and conventional PCR. For conventional non-modified maize kernels, RT-PCR wasn't conducted.

Consequently, due to the obtained results, limit of detection was clarified as 2% for conventional PCR and 0.1% for Real-Time PCR. Since the 0.5% CRM was used during quantification as a lowest concentration, limit of quantification was accepted as %0.5 for Real Time PCR.

Obtained results for each sample were summarised in Table 3.14.

Table 3.14. General interpretation of results

Samples	Sample sort	Plant specific PCR		Screening PCR				Event specific PCR		
		Zein		CaMV		NOS		Kan	Bt11	
		Zein by conventional PCR	Zein via RT-PCR	CaMV via conventional PCR	CaMV via RT-PCR	NOS via conventional PCR	NOS via RT-PCR		Bt11 via conventional PCR	Bt11 via RT-PCR
%0	CRMs	+	+	-	-	-	-	-	-	-
%0,1		+	+	-	+	-	+	-	-	/
%0,5		+	/	-	/	-	/	-	-	+
%1		+	/	-	/	-	/	-	-	/
%2		+	/	+	/	+	/	-	+	/
%5		+	/	+	/	+	/	-	+	+
1	Food	+	+	+	+	+	-	-	+	+
2		+	+	+	+	+	-	+	-	-
3		+	+	-	+	-	+	-	-	-
4		+	+	+	+	+	+	-	-	-
5		+	+	+	+	-	+	-	-	+
6	Feed	+	+	-	+	+	-	-	+	+
7		+	+	+	+	+	-	+	-	+
8		+	+	+	+	+	+	-	+	+
9		+	+	+	+	+	+	-	+	+
10		+	+	+	+	-	-	-	+	+
11	Maize Flour	+	+	+	+	-	+	-	-	-
12		+	+	-	+	-	-	-	+	/
13		+	-	+	+	-	-	+	-	-
14		+	+	-	-	-	-	-	-	/
15		+	+	-	-	-	-	-	-	/

		Plant specific PCR		**Screening PCR**				**Event specific PCR**		
Samples	Sample sort	Zein		CaMV		Nos		Kan	Bt11	
		Zein by conventional PCR	Zein by RT-PCR	CaMV via conventional PCR	CaMV via RT-PCR	Nos via conventional PCR	Nos via RT-PCR		Bt11 via conventional PCR	Bt11 via RT- PCR
16	Maize starch	+	+	-	+	-	+	-	/	/
17		+	+	-	-	-	-	-	/	/
18		+	+	-	-	-	-	-	/	/
19		+	-	-	-	-	-	-	/	/
20	Corn flakes	+	-	-	-	-	-	-	/	/
21		+	+	-	-	-	-	-	/	/
22		+	+	-	-	-	-	-	/	/
23	Maize chips	+	+	-	-	-	-	-	/	/

CHAPTER IV

CONCLUSION

In Turkey, maize is one of the most widely used food not only as a raw material but also as an ingredient for food processing. Most of the maize kernels have been imported from South Africa, Brazil, Argentina and the USA. Till now, there is no related data about consumption of genetically modified maize foods in Turkey. In this study, detection and quantification of collected samples from Turkey food market were conducted via DNA based techniques.

By means of real time PCR and conventional PCR, genetically manipulated samples were determined. Advantages of Real time PCR over conventional PCR techniques in detection of genetically modified foods were emphasised. In most of the processed samples as maize chips, pop corn, corn flakes , no detection was found whereas in addition to raw material, transgenes were detected in maize starch and maize flour. It seems that in non-detected samples, concentrations of genetically modified DNA may below the detection limits or they may be non-GM. However these limitations can be overcome and limit of detection may be diminished. Using commercial DNA isolation kits instead of CTAB method may cause isolating DNA with a better quality. The amount of used Taq Polymerase can be increased. The beginning amount of homogenised sample also hamper the quantification and detection. Starting with 1 kg of homogenised sample instead of 50 g homogenised sample decrease the limit of detection. The more the initial starting material , the better the quantification. Food DNA was damaged due to the processing. It should be emphasised that generating short PCR products gives higher sensitivity in the detection of processed foods.

The study focused on Bt11 maize line which was approved for food use in EU, UK, Switzerland, Japan, Canada and Argentina, Australia, USA and South Africa. However in the national level discussions for the consumption of Bt11 continue for cultivation.

This study obviously pointed out that, there exist genetically modified maize on Turkey food market. Uncontrolled importation and distribution bring about the presence of GM maize lines in Turkey food market. The related regulations and legislations have been still in progress. Consumer purchasing decisions should be respected and mandatory GMO labelling need to be implemented at once. In all over the world, it is likely that general attitude regarding genetic modification will change with time, but whether this change will go towards a more positive or negative stance is still unknown.

There is still a need for more precise research to clarify some contradictory matters regarding GM foods. Moreover, nutritional and food safety aspects of genetically modified plants should be taken into account . To prevent unintended effects on human, animal health and environment, research laboratories where the genetically modified foods subjected to analysis should be established.

This study will help to pay an attention the uncontrolled consumption of GM foods in Turkey and result in the GM Foods to be objected of specific regulation.

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APPENDIX A

CHEMICALS AND SUPPLIERS

Table A.1. Chemicals and suppliers

CHEMICALS	SUPPLIERS
Agarose	Applichem
Sodium Chloride (NaCl)	Merck
Sodium Hydroxide (NaOH)	Merck
Tris	Sigma
Ethylenediaminetetraacetic (EDTA)	Sigma
Hydrogen chloride (HCl)	Applichem
Sodium Acetate (NaAc)	Applichem
Polyvinylpyrrolidone (PVP)	Sigma
Hydrogen Peroxide (H_2O_2)	Sigma
Ficol 400	Sigma
Bromphenol Blue	Sigma
Ethidium Bromide (EtBr)	Sigma
Dimethyl Sulfoxide (DMSO)	Applichem
Glacial Acetic Acid	Applichem
Sodium Dodeyl Sulphate(SDS)	Merck
Ethanol (EtOH)	Delta Kimya
Isopropanol	Delta Kimya
Chlorophorm: Isoamylalcohol	Applichem
Hexadecyltrimethyl-Ammonium Bromide (CTAB)	Applichem

APPENDIX B

BUFFERS AND SOLUTIONS

1.Solutions for DNA isolation

1.1. Hexadecyltrimethyl-Ammonium Bromide (CTAB) Buffer

CTAB	20g/l
NaCl	1.4 M
Tris HCl	100 mM
EDTA	20 mM

Bring the last volume to 1 liter with dH_2O. Adjust the pH to 8 and autoclave (121 ^oC, 15 min).

1.2. CTAB Precipitation Buffer

CTAB	5g/l
NaCl	0.04 M

Bring the last volume to 1 liter with dH_2O. Adjust the pH to 8 and autoclave (121 ^oC, 15 min).

1.3. 1.2 M NaCl

NaCl	70g
dH_2O	1 liter

Dissolve in 1000 ml dH_2O and autoclave (121 ^oC, 15 min)..

1.4. Washing Buffer

dH_2O 300ml
Pure EtOH 700ml

2. Buffers for Electrophoresis

2.1. Electrophoresis Buffer 50X TAE (Tris-Acedic acid–EDTA)(Maniatis, 1989)

2M Tris
1M Acedic Acid
100 mM Na_2EDTA

48. 44g, 11.8g and 7.45g respectively for 200 ml solution were dissolved in dH_2O and Ph was adjusted to 8.0. The solution was diluted 50 times before using.

2.2 Loading Buffer for Agarose Gel Electrophoresis (Maniatis, 1989)

0.25 % Bromophenol Blue
0.25 % Xylene cyanol
15 % Ficol 400
40% (w/v) Sucrose

Dissolved in dH_2O.

2.3. Ethidium Bromide Solution (Maniatis, 1989)

10 mg/ml EtBr was dissolved in dH_2O.

APPENDIX C

ENZYMES, MARKERS AND REAGENTS

Table C.1. Enzymes, markers and reagents

ENZYMES, MARKERS AND REAGENTS	SUPPLIER
DNA Taq Polymerase	MBI Fermentas
Ribonuclease A (RNAse)	MBI Fermentas
Proteinase K	MBI Fermentas
DNA ladder	MBI Fermentas
Deoxynucleotide Triphosphates (dNTPs)	MBI Fermentas
Lambda DNA	MBI Fermentas
PstI	MBI Fermentas

APPENDIX D

HOMOLOG REGIONS AND PRIMER BINDING SITES OF SEQUENCES

1. Primer binding site for plant specific primer , Ze-01 and Ze-02.

Annealing sites of Ze-01 and Ze-02 primer sets were illustrated as red colour. The sequence between primer binding sites was amplified PCR product.

```
1111 ctctaggaag caaggacacc accgccatgg cagccaagat gcttgcattg ttcgctctcc   1170
1171 tagctctttg  tgcaagcgcc actagtgcga cccatattccaggcacttg ccaccagtca    1230
1231 tgccattggg taccatgaac ccatgcatgc agtactgcat gatgcaacag gggcttgcca   1290
1291 gcttgatggc gtgtccgtcc  ctgatgctgc agcaactgtt ggccttaccg  cttcagacga  1350
1351 tgccagtgat gatgccacag atgatgacgc ctaacatgat gtcaccattg atgatgccga   1410
1411 gcatgatgtc accaatggtc ttgccgagca tgatgtcgca aataatgatg ccacaatgtc   1470
1471 actgcgacgc cgtctcgcag attatgctgc aacagcagtt accattcatg ttcaacccaa    1530
```

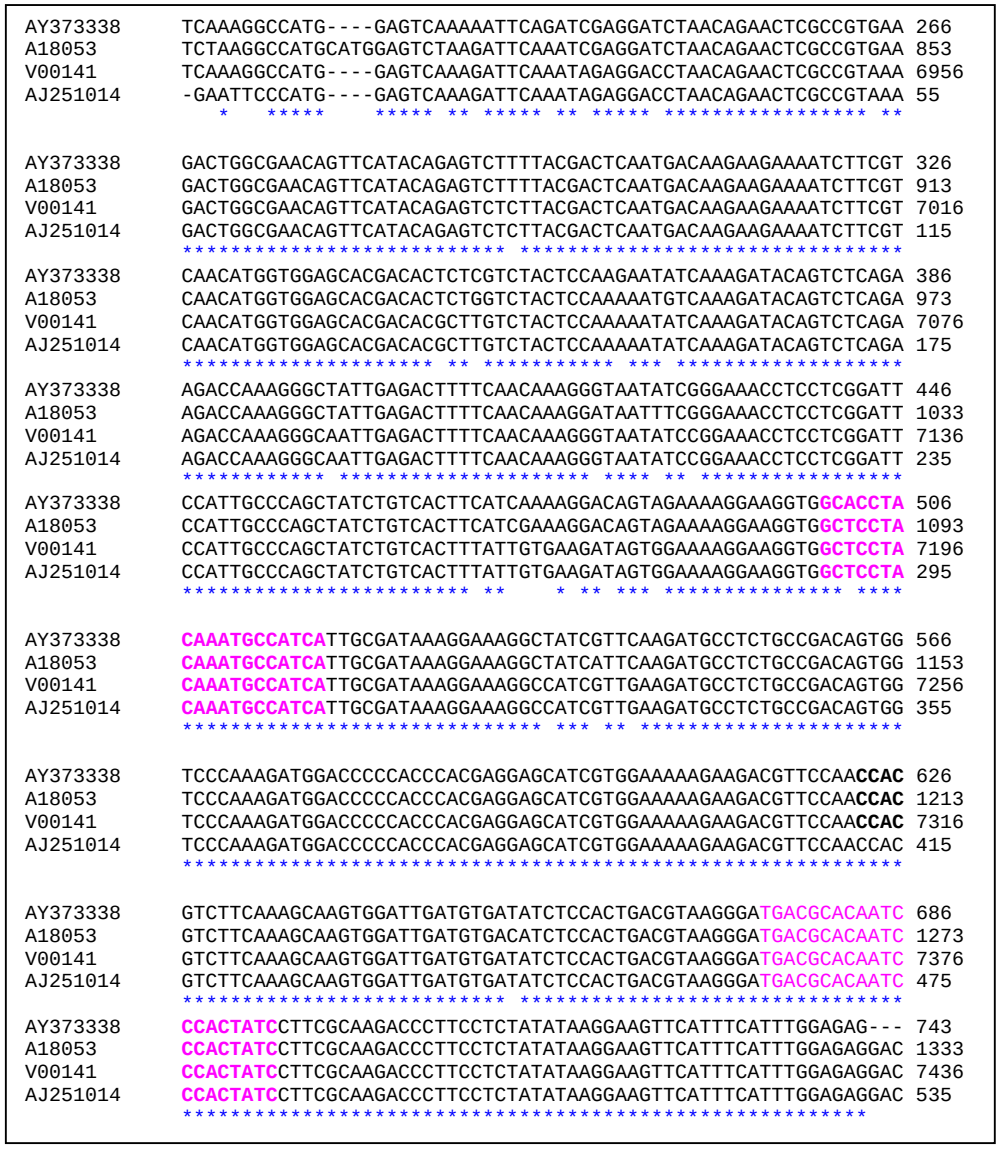
Figure D.1. Primer binding sites of zein primer sets

3. Primer binding sites for screening primers

- **Primer binding sites and sequence homology among 35S promoter sequences**

Selected sequences were aligned and homolog regions were checked. Primer annealing sites for 35S-1 and 35S-2 primer sets represented as red colour.

Figure D.2. Primer binding sites of 35S primer pairs



❖ Primer binding sites and sequence homology among Nos terminator sequences

Selected sequences were aligned and homolog regions were checked. Primer annealing sites for nos specific primer sets represented as red colour.

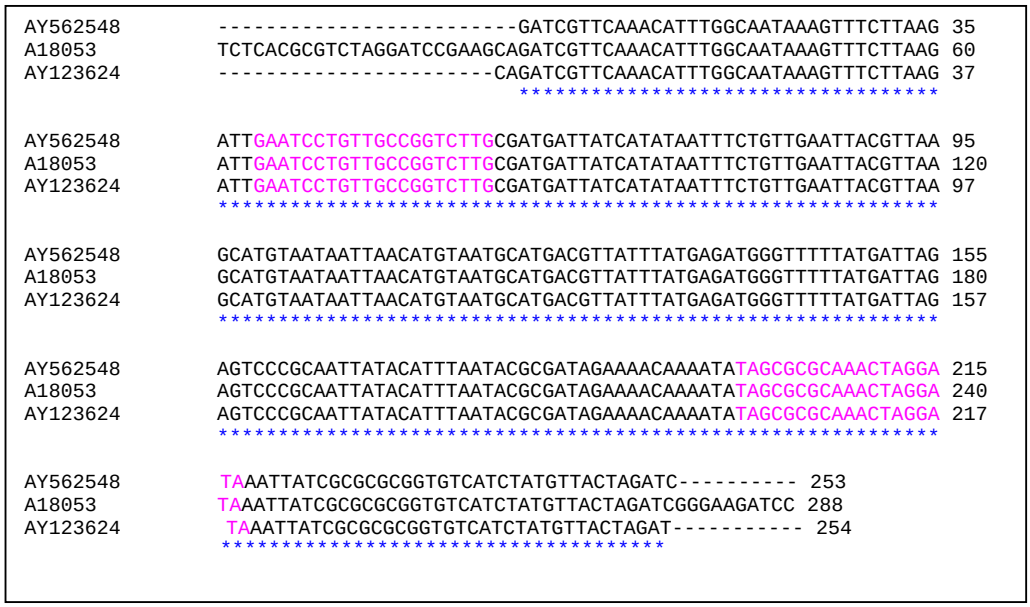


Figure D.3. Primer binding sites of Nos primer pairs

❖ Primer binding sites and sequence homology among Kanamycin resistant sequences

Selected sequences were aligned and alignments were checked. Primer annealing sites for kanamycin resistance primer sets represented as red colour.

```
AF274974        TCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG 300
AF274586        TCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG 278
AF485783        TCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG 92
AY456412        TCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG 92
AY159034        TCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG 92
AF274975        TCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG 164
                ************************************************************


AF274974        CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGAC 360
AF274586        CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGAC 338
AF485783        CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGAC 152
AY456412        CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGAC 152
AY159034        CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGAC 152
AF274975        CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGAC 224
                ************************************************************

AF274974        CGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGC 420
AF274586        CGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGC 398
AF485783        CGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGC 212
AY456412        CGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGC 212
AY159034        CGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGC 212
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AF274975        CGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGC 284
                ************************************************************

AF274974        CACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTG 480
AF274586        CACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTG 458
AF485783        CACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTG 272
AY456412        CACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTG 272
AY159034        CACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTG 272
AF274975        CACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTG 344
                ************************************************************

AF274974        GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 540
AF274586        GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 518
AF485783        GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 332
AY456412        GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 332
AY159034        GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 332
AF274975        GCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGA 404
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AF274974        GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 600
AF274586        GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 578
AF485783        GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 392
AY456412        GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 392
AY159034        GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 392
AF274975        GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG 464
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AF274974        CCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 660
AF274586        CCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 638
AF485783        CCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 452
AY456412        CCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 452
AY159034        CCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 452
AF274975        CCCATACGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG 524
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AF274974        TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 720
AF274586        TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 698
AF485783        TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 512
AY456412        TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 512
AY159034        TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 512
AF274975        TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTT 584
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AF274974        CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 780
AF274586        CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 758
AF485783        CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGATGATCTCGTCGTGACCCATGGCGATGC 572
AY456412        CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGATGATCTCGTCGTGACCCATGGCGATGC 572
AY159034        CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 572
AF274975        CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGC 644
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AF274974        CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 840
AF274586        CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 818
AF485783        CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 632
AY456412        CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 632
AY159034        CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 632
AF274975        CTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG 704
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AF274974        GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 900
AF274586        GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 878
AF485783        GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 692
AY456412        GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 692
AY159034        GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 692
AF274975        GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGA 764
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AF274974        GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 960
AF274586        GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 938
AF485783        GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 752
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AY456412        GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 752
AY159034        GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 752
AF274975        GCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTC 824
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AF274974        GCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 1003
AF274586        GCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 981
AF485783        GCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 795
AY456412        GCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 795
AY159034        GCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 795
AF274975        GCAGCGGATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 867
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Figure D.4. Primer binding sites of kanamycin primer pair

APPENDIX E

FLOWCHARTS OF PROCESSED FOODS

❖ **Production of Corn Flour**

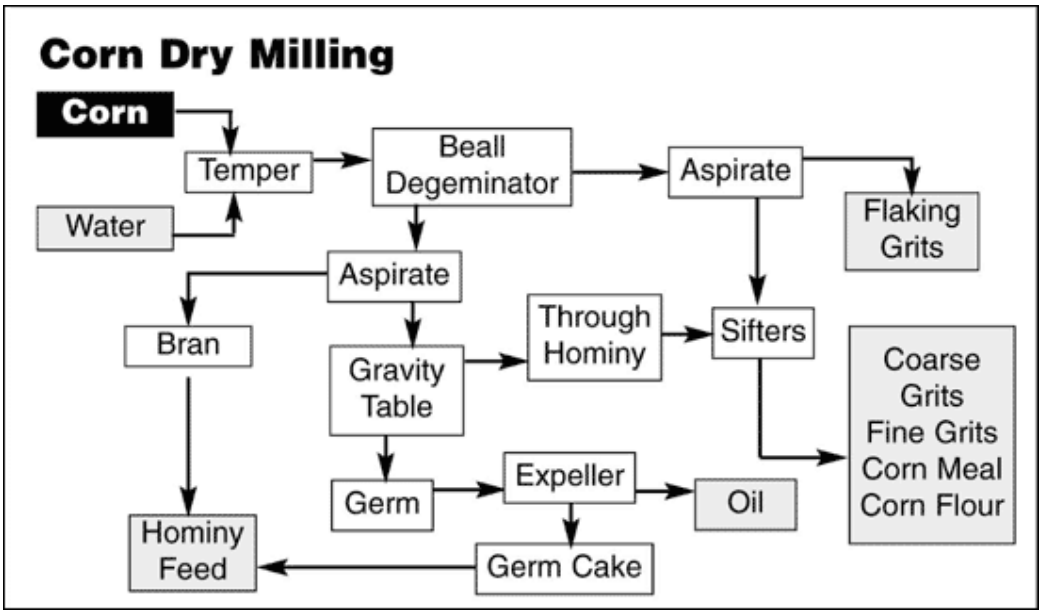

Figure E.1. Flowchart of corn flour processing (http://www.iowacorn.org/cornuse/cornuse_7.html#corndry, 17-09-2004)

❖ **Production of corn starch**

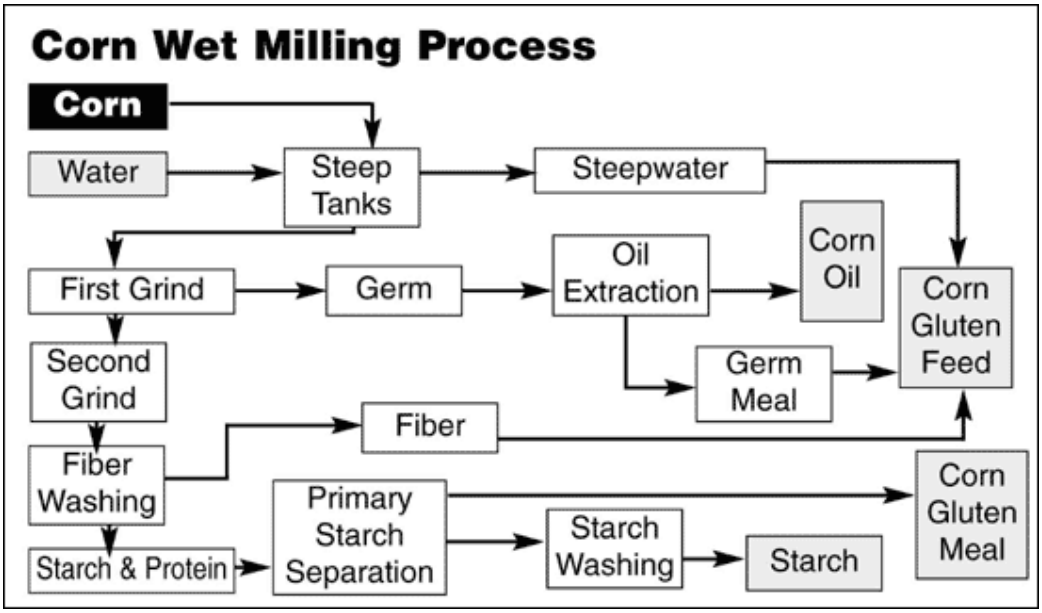

Figure E.2. Flowchart of corn starch processing, (http://www.iowacorn.org/cornuse/cornuse_7.html#corndry, 17-09-2004)

APPENDIX F

TOTAL FLUORESCENCE SIGNALS OF RT-PCR FOR SCREENING

A-Fluorescence Signals for the NOS terminator screening

1-Total signals of first run nos terminator screening

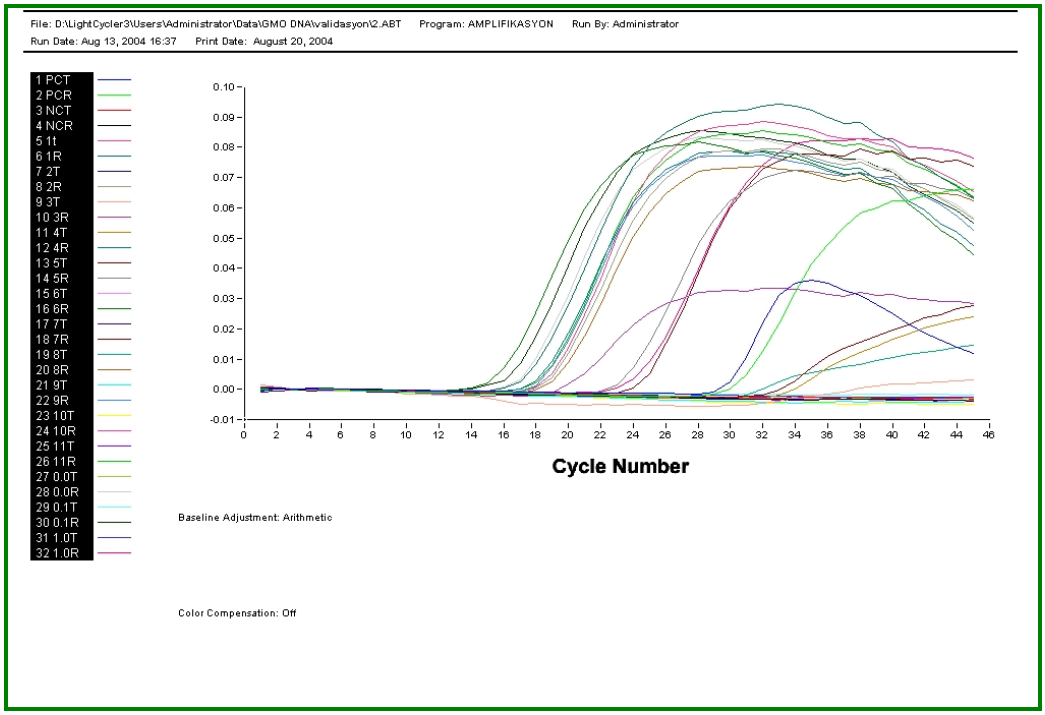

Figure F.1. Total signals of first run nos terminator screening

2-Total signals of second run nos terminator screening

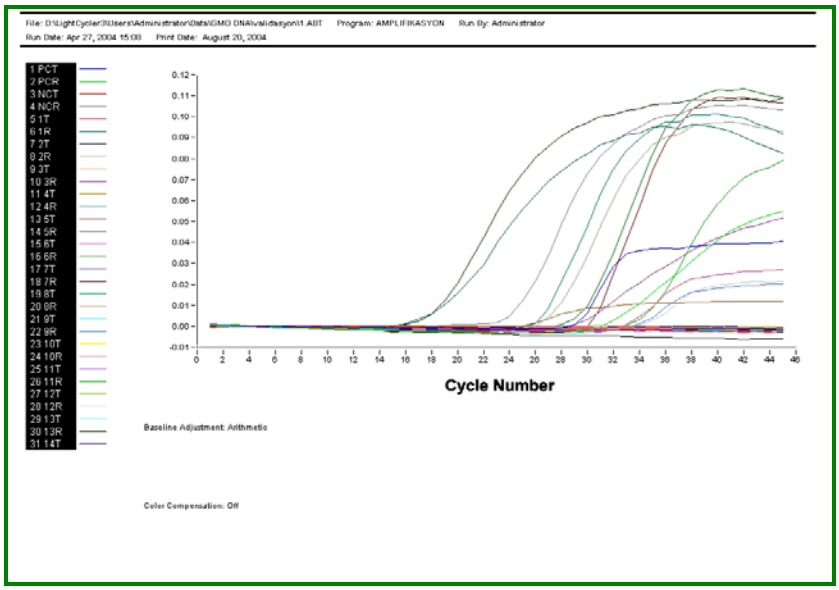

Figure F.2. Total signals of second run nos terminator screening

3-Total signals of third run nos terminator screening

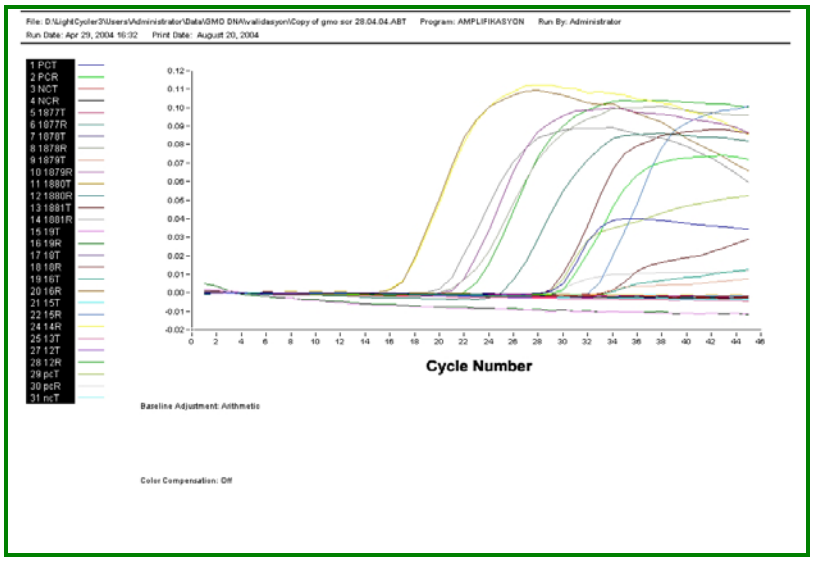

Figure F.3. Total signals of third run nos terminator screening

B-Fluorescence Signals for the CaMV 35S promoter screening screening

1-First run total signals of 35S promoter screening

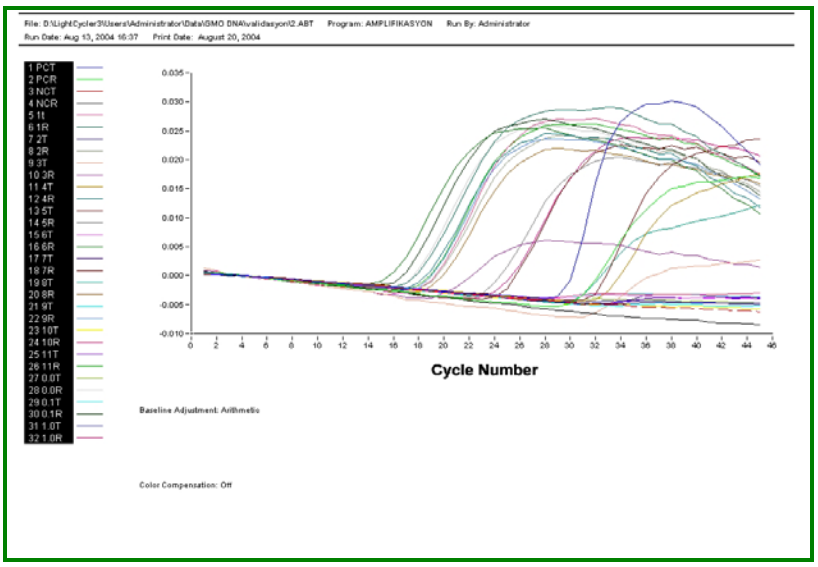

Figure F.4. First run total signals of 35S promoter screening

2-Second run total signals of 35S promoter screening

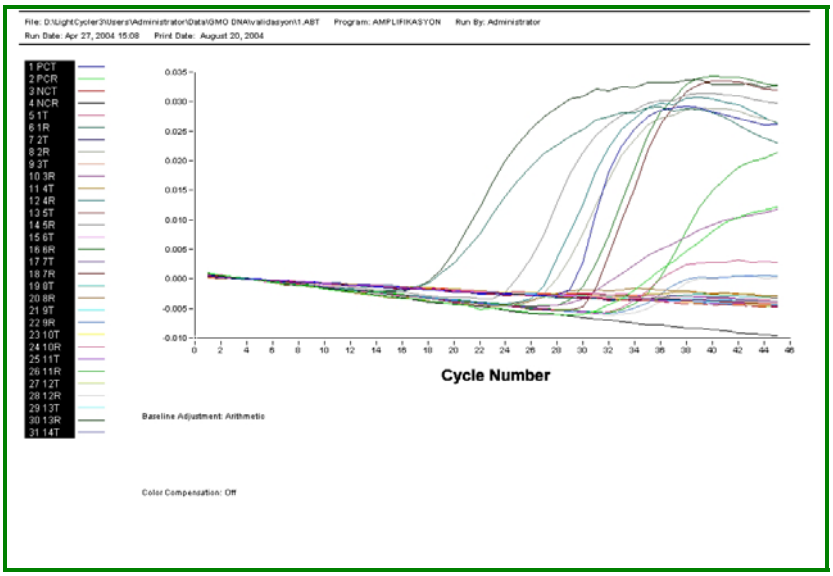

Figure F.5. Second run total signals of 35S promoter screening

3-Third run total signals of 35S promoter screening

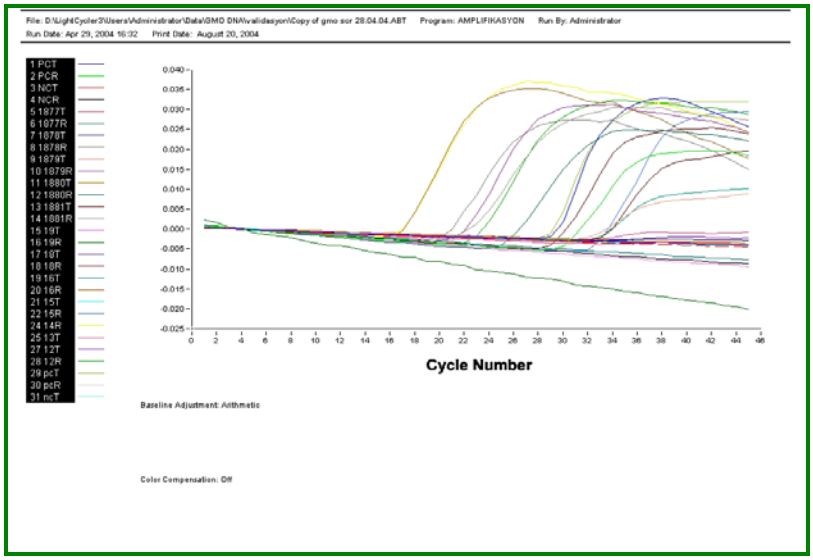

Figure F.6. Third run total signals of 35S promoter screening

APPENDIX G

THE LOCATION OF THE UNKNOWN SAMPLES ON STANDARD CURVE

The blue points represents the positive controls whereas red point represents the unknown analysed samples.

❖ **Standard curve for ivs**

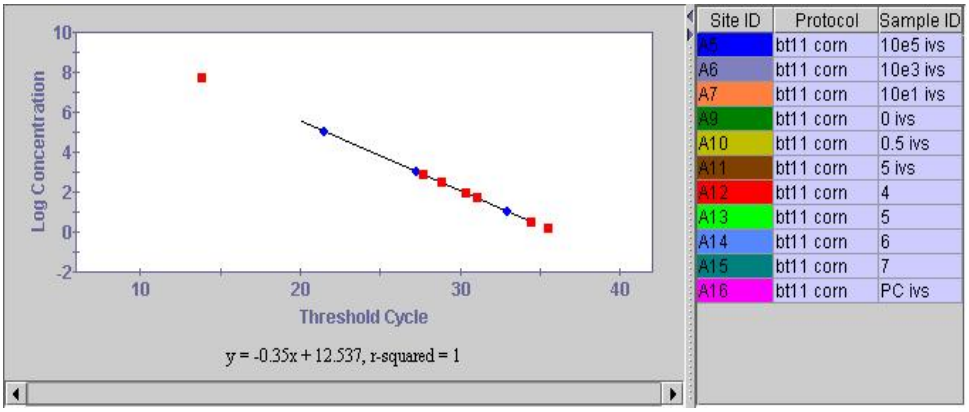

Figure G.1. Standard curve for ivs

❖ **Standard curve for zein**

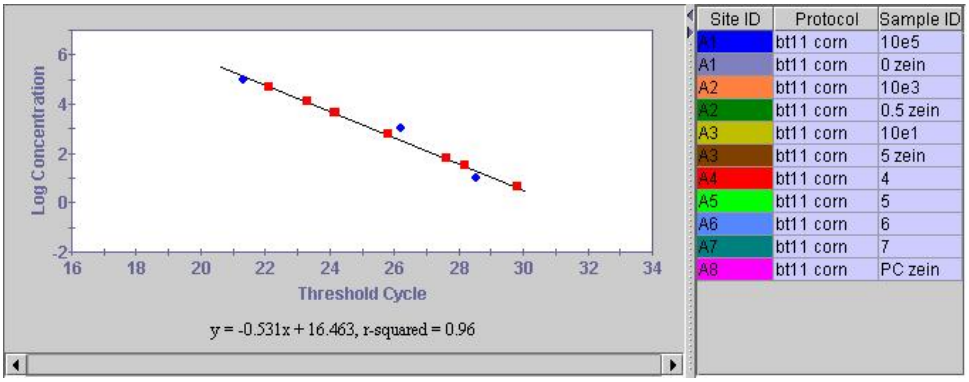

Figure G.2. Standard curve for zein gene

APPENDIX H

HISTORY OF EUROPEAN GM LEGISLATION 1990-2003

Table H.1. History of EU GM legislation 1990-2003 (Lee and Carson, 2004)

Year	Title	Contents
1990	**Directive 90/219**	**The contained use of GM micro-organisms.**
1990	**Directive 90/220**	**The deliberate release into the environment of GMOs (repealed)**
1991	Decision 91/274	Legislation referred to in Art. 10 of Directive 90/220
1991	Decision 91/448	Concerning guidelines for classification referred to in Directive 90/219 (amended by Decision 96/134)
1991	Decision 91/596	The Summary Notification Information Format referred to in 90/220, art 9 on the deliberate release into the environment of GMOs.
1992	Decision 92/146	Summary notification information format referred to in Art.12 of Directive 90/220.
1993	Decision 93/572	The placing on the market of a product containing GMOs pursuant to Article 13 Directive 90/220.
1993	Decision 93/584	Establishing the criteria for simplified procedures concerning the deliberate release into the environment of genetically modified plants pursuant to Article 6(5) Directive 90/220.
1994	Directive 94/51	Adapting to technical progress for the first time 90/220 on the deliberate release into the environment of GMOs.
1994	Decision 94/211	Amending Decision 91/596 concerning 1994the summary notification information format referred to in Art.9 of Directive 90/220
1994	Decision 94/385	The placing on the market of a product consisting of a GMO, seeds of herbicide-resistant tobacco variety ITB 1000 OX, pursuant to 90/220, Art 13.
1994	Decision 94/730	Establishing simplified procedures concerning the deliberate release into the environment of genetically modified plants pursuant to 90/220, art 6(5).
1996	Decision 96/134	Amending Decision 91/448 on Directive 90/219 guidelines for classification.
1996	Decision 96/158	The placing on the market of a product consisting of a genetically modified organism, hybrid herbicide-tolerant swede-rape seeds (Brassica napus L. oleifera Metzq. MS1BN x RF1Bn) pursuant to 90/220.

Year	Title	Content
1996	Decision 96/281	**The placing on the market of GM soya beans (Glycine max L) with increased tolerance to the herbicide glyphosate, pursuant to Directive 90/220. This is the marketing consent for Monsanto Round-up Soya.**
1997	**Regulation 258/97**	**Novel Foods and Novel Foods Ingredients.**
1997	Decision 97/392	**The placing on the market of GM swede-rape (Brassica napus L.oleifera Metzg. MS1, RF1) pursuant to Directive 90/220.**
1997	Decision 97/393	The placing on the market of GM swede-rape (Brassica napus L.oleifera Metzg. MS1, RF2), pursuant to Directive 90/220.
1997	**Regulation 1813/97**	**The compulsory indication on the labelling of certain foodstuffs produced from GMOs in addition to the particulars required in food labelling laws (repealed in 1998).**
1997	**Directive 97/35**	**Compulsory labelling of all new agriculture producing or containing GMOs notified under**
1997	Decision 97/549	The placing on the market of T102-test (Streptococcus thermophilus T102) pursuant to Directive 90/220
1998	**Directive 98/81**	**Amending Directive 90/219/EEC on the contained use of genetically modified micro-organisms1998 OJ L330/13**
1998	Decision 98/291	The placing on the market of GM spring swede rape (Brassica napus L. ssp Oleifera) pursuant to Directive 90/220.
1998	Decision 98/292	The placing on the market of GM maize (Zea mays L. line Bt-11) pursuant to Directive 90/220.
1998	Decision 98/293	The placing on the market of GM maize (Zea mays L. line T25), pursuant to 90/220. 1998 Decision 98/294 The placing on the market of GM maize (Zea mays L. line MON 810) pursuant to Directive 90/220.
1998	Decision 98/613	Concerning a draft Decree of Austria on the identification of genetically modified additives and flavourings used as food ingredients
1998	**Regulation 1139/98**	**The compulsory indication of the labelling of GM Soya/Maize foodstuffs, repealing Regulation 1813/97.**
2000	Decision 2000/608	Concerning the guidance notes for risk assessment outlined in Annex III of Directive 90/219
2000	**Regulation 49/2000**	**The *de minimis* level for food accidentally contaminated with GM soya or maize. The labelling requirements under Regulation 1139/89 not applying if the proportion is no higher than 1% of the food ingredient being considered.**
2000	**Regulation 50/2000**	**The labelling of foodstuffs and food ingredients containing additives and flavourings that have been genetically modified or have been produced from GMOs.**
2000	Decision 2000/608	The guidance notes for risk assessment outlined in Directive 90/219, Annex III on the contained use of GM micro-organisms..
2001	Decision 2001/204	Supplementing Directive 90/219 as regards the criteria for establishing the safety for human health and the environment, of types of GMOs
2001	**Directive 2001/18**	**The deliberate release into the environment of GMOs and repealing Directive 90/220.**
2002	Decision 2002/623	Establishing guidance notes supplementing Annex II to 2001/18
2002	Decision 2002/811	Establishing guidance notes supplementing Annex VII to 2001/18 on the deliberate release into the environment of GMOs and repealing Directive 90/220.
2002	Decision 2002/812	Establishing pursuant to Directive 2001/18 summary information format relating to the placing on the market of GMOs as or in products.
2002	Decision 2002/813	Establishing, the summary notification information format for notifications concerning the deliberate release into the environment of GMOs for purposes other than for placing on the market
2003	**Regulation 1829/03**	**New Regulation on GM Food and Feed.**
2003	**Regulation 1830/03**	**New Regulation on GM Traceability and Labelling.**
2003	**Regulation 1946/03**	**New Regulation on Transboundary Movement.**

SUMMARY OF EU CONSUMER PROTECTION INFORMATION

Table H.2. Summary of EU consumer protection information (Lee and Carson, 2004)

Year	Main Legislation	Laws introduced	Main Gaps identified
Pre-1997	· Directive 90/219 · Directive 90/220	· Contained use of GMOs · Deliberate release of GMOs	· No labelling of foodstuffs containing GMOs · Note: weaknesses in both Directives addressed during 1998 - 2002
1997	· Directive 97/35 · Regulation 258/97 · Regulation 1813/97	· Compulsory labelling of GMOs notified under 90/220 · Review and approval of Novel Foods introduced AFTER 15 May 1997	· Novel Foods procedure does not apply to food on market PRE 15 May 1997 · Approved GM soya/maize not covered by Novel Foods (addressed in Reg. 1813/97) · Novel Foods does not apply to additives, flavourings or extraction solvents · No de-minimis threshold · No labelling rules on GM additives and flavourings
2003	· Regulation on Food and Feed 1829/2003 · Regulation on Traceability and Labelling 1830/2003 · Regulation on Transboundary Movement 1946/2003	· Compulsory labelling of GM food and feed regardless of detectability · Introduces labelling of animal feed derived from a GMO. · Labelling of GM additives and flavouring regardless of detectability. · Centralised community procedure involving EFSA .Harmonised framework for tracing and identifying GMOs, GM food and feed at all stages · Development of unique identifiers · System for notification and exchange of information, to implement Cartagena Protocol.	· Limited to "from" a GMO so no labelling of foods/feed "with" a GMO such as chymosin · Labelling of GM processing aids not covered · No labelling for GM fed animal products
2004+	Unknown	None at present except the Commission has mentioned addressing the labelling of GM enzymes	. Labelling for all GM processing aids? · Labelling for GM fed animal products? · Labelling for foods produced from or with help of GM enzyme?