ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

P35S, TNOS AND PFMV TARGETED MULTIPLEX PCR USING A SINGLE DYE

M.Sc. THESIS

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Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology Master Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

TEK BOYA KULLANARAK P35S, TNOS VE PFMV HEDEFLİ ÇOKLU PZR

YÜKSEK LİSANS TEZİ

Deniz Gülbin TAN
521101104

İleri Teknolojiler Anabilim Dalı

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Tez Danışmanı: Doç. Dr. Gizem DİNLER DOĞANAY

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Deniz Gülbin TAN, a **M.Sc.** student of ITU **Graduate School of science engineering and technology** student ID 521101104, successfully defended the **thesis** entitled "**P35S, TNOS AND PFMV TARGETED MULTIPLEX PCR USING A SINGLE DYE**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Doç. Dr. Gizem DİNLER DOĞANAY** Istanbul Technical University	.....
Co-advisor :	**Dr. Mustafa KOLUKIRIK**	.....
Jury Members :	**Assoc. Prof. Dr. Eda Tahir TURANLI** Istanbul Technical University	.....
	Prof. Dr. Bahar İNCE Boğaziçi University	.....
	Prof. Dr. Dilek KAZAN Marmara University	.....

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To my family,

FOREWORD

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Molecular biolog

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ABBREVIATIONS

Bar : Phosphinotricin-Nacetyltransferases from S. hygroscopicus
Bla : Ampicillin
Bt : Biotech
Bt : Bacillus thuringiensis
CaMV : Cauliflower mosaic virus
C-PCR : Competitive PCR
Microarray : Microarray
ELISA : Enzyme linked immunosorbent assay
Epsps : 5-enolpyruvylshikimate-3-phosphate synthase
FRET : Fluorescence resonance energy transfer
GM : Genetically modified
GMOs : Genetically modified organisms
HRM : High resolution melting
HT : Herbicide tolerance
IR : Insect resistance
LNA : Locked nucleic acid
LUX : Light upon extension
MPIC : Microdroplet PCR implemented capillary gel electrophoresis
nptII : Neomycin/ kanamycin
p35S : Cauliflower mosaic virus 35 S promoter
pAct : Rice actin promoter
PAGE : Polyacrylamide gel electrophoresis
Pat : Phosphinotricin-Nacetyltransferases from S. viridochromogenes
PCR : Polymerase chain reaction
PEG : Polyethylene glycol
pFMV : Figworth mosaic virus 35S promoter
pNOS : Agrobacterium tumefaciens nopaline synthase promoter
pUbiZM : Maize ubiquitine promoter
S-ELISA : Sandwich enzyme linked immunosorbent assay
t35S : Cauliflower mosaic virus 35 S terminator
T-DNA : Transfer DNA
Taq : *Thermus aquaticus*
Ti : Tumor inducing
T_m : Melting temperature
tNOS : Agrobacterium tumefaciens nopaline synthase terminator
QPCR : Real-time PCR

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P35S, TNOS AND PFMV TARGETED MULTIPLEX PCR USING A SINGLE DYE

SUMMARY

Food plants that are being produced or modified by genetic engineering techniques are conventionally named as genetically modified (GM) crops or genetically modified organisms (GMOs). The investigations have revealed different results on the risks of GMOs on human health and the environment. The regulatory need to monitor and verify the presence and the amount of GM varieties in crops and products has increased with the release of GM crops and products in the markets worldwide. Therefore, there is a need to develop reliable, quick and cost-effective methods for the detection of GM varieties in crops and their products.

Screening for the GMO promoters or terminators is usually the first step for GMO analysis. The subsequent event specific qualitative and quantitative GMO analyses must be carried on the GMO positive samples to ensure that the detected GMOs were not originated from the contaminations. This has a substantial importance in countries where the quantitative threshold levels were defined for labeling of the GM products.

In this study, we developed a multiplex QPCR methodology using a single high resolution melting dye to simultaneously detect Cauliflower Mosaic Virus 35S (35S) promoter, *Agrobacterium tumefaciens* Nopalin Synthase (NOS) terminator and Figworth mosaic virus 35S (FMV) promoter, which are contained in more than 99% of the GMO events. Discrimination between the different PCR products was based on the differences in melting temperatures of the target DNAs. We also developed an enzyme free DNA extraction methodology for food samples to shorten the total analysis time necessary for the screening of these elements.

High quality DNA is necessary to obtain sensitive and efficient results in PCR based methodologies. In this study, we tried 5 different silica column based DNA extraction protocols on soybean and maize samples to obtain DNA with high quantity and quality. Three of the protocols were based on enzymatic steps whereas the other two methods were completely based on the chemical and physical cell disruption methodologies. In all of the methodologies, guanidium thiocyanate was used for PCR inhibitor inactivation and as a catastrophic agent for DNA binding.

The current methodologies of DNA extraction for GMO detection must result in at least 1.5 µg DNA with $A_{260/280}$ ratios between 1.6 and 2.0. $A_{260/280}$ ratios of DNA extracts from all of the developed methods were in the desired range. All of the protocols were resulted in DNA amounts higher than 15 µg DNA, which is at least 10x higher than the minimal limit. The best results in terms of DNA concentration were obtained from the protocols that include bead beating and CTAB treatment.

Since proteins absorb at 280 nm, the ratio $A_{260/280}$ is used to estimate the presence of the proteins in DNA extracts. On the other hand, the presence of other types of PCR inhibitors such as carbohydrates, phenols, aromatic compounds and heavy metals may also affect the PCR results. To comparatively evaluate effect of the DNA quality obtained by different protocols on the QPCR efficiency, the same amount of template DNAs were used in QPCR. The universal plant chloroplast DNA targeted PCR primers were used in real time PCR trials. The obtained C_t values indicated the presence of PCR inhibitors because DNA concentrations and purities were the same for all the diluted templates obtained from different protocols. All of the templates were resulted in plant chloroplast DNA specific melting temperatures (T_m). Threshold cycle (Ct) values obtained using the protocols that include bead beating and CTAB treatment were approximately 2 cycles lower than the other protocols. This showed that these two protocols were more successful in eliminating the PCR inhibitors. The difference between these two protocols was the inclusion of proteinase K treatment step. To reduce the cost and total time necessary for the DNA isolation, we chose the protocol without proteinase K treatment.

FMV, NOS, 35S positive reference food samples were supplied by the accredited food control laboratories. Extracted DNAs from FMV, NOS, 35S positive food samples were amplified by using the target specific primer pairs. Melting curve analysis was performed after the amplification cycles and T_m of the targeted PCR products were calculated. The target specific melting peaks were obtained at 73 ± 0.38˚C for NOS, 80˚C ± 0.28˚C for FMV, 82.26 ± 0.29˚C for 35S and 82 ± 0.33˚C for plant specific reactions. It is generally accepted that the T_m obtained with Evagreen QPCR could vary between 0.5 and 1 ˚C for the same amplicon. In this study, the standard deviations were lower than 0.4 ˚C. In addition, all of the Evagreen QPCR reactions generated a single specific signal without major additional amplification products.

QPCR quantification standards were prepared using the purified PCR products from the reference samples. Serial dilutions were done to obtain standard samples containing 10^0-10^{10} copies of the targeted gene. In order to obtain the limit of detection (LOD), soybean samples that contain 1-100 copies of 35S and NOS per gr of the sample, and maize samples that contain 1-100 copies of FMV per gr of the sample were prepared. The limits of detection were 1 gene copy/gr food sample for the 35S, NOS and FMV targeted methodologies. On the other hand, since the standard mixtures were not obtained from a reference food control laboratory, the detected LODs were rough estimations of the real LODs.

A DNA mixture of the 35S, NOS, FMV genes were prepared to test the specificity of the primers. The DNA mixture was amplified via QPCR with each specific primer pair. The specificity of the QPCR reactions was examined via sequencing of the each amplified PCR product. The results showed that the amplified sequences have at least 99% similarity to the intended targets.

The same amounts of the different DNA templates were added to the initial duplex QPCRs trials. Favored DNA templates, which resulted in more abundant PCR products in duplex reactions, were determined via melting curve analysis. The FMV templates resulted in more PCR products. The 35S templates were favored in PCRs that contained the 35S and NOS templates.

The subsequent trials were carried out till only one type of T_m peak was obtained to determine the effect of different initial template amounts on the duplex QPCRs. The

overall results showed that; two different T_m peaks were not obtained under 1/100 relative template concentrations but two different T_m peaks were obtained for each target above 1/100 relative template concentrations.

After the successful binary mixture trials, triple mixture was prepared using 1000 copies of the each reference sample. Triplex QPCR trials were carried out to show that 3 primer pairs can work together in the multiplex QPCR and do not form non-specific PCR products or primer dimers. The triple combinations were applied to 1/1/1 relative copy number ratios of the reference samples. The NOS, FMV and 35S specific multiplex QPCR resulted in 3 different melting peaks. The melting peak corresponding to NOS, FMV and 35S targets were observed at 73.04±0.13˚C, 80.21±0.10˚C and 82.15±0.08˚C, respectively. No additional amplification was observed in the multiplex reactions.

Since plant DNAs will always be the dominant target in GMO screening reactions, plants DNAs were not included in the binary and triple DNA mixtures to increase the detection sensitivities of the FMV, 35S and NOS targets. Plant specific QPCRs were carried out in GMO screening reactions as a positive PCR amplification control.

The raw and processed food samples, which were already analyzed by the accredited food control laboratories, were re-analyzed using the developed methodology. Total of 96 samples that include meatballs, soybean oil, soybean meal, corn, corn oil, tallow oil, cat and dog foods, chocolate, baklava and bread varieties were analyzed. Our results were in 100% accordance with the results obtained by the accredited food control laboratories.

This study showed that multiple detection of 35S, NOS and FMV is possible using a single HRM dye. We also showed that it is possible to extract high quality DNA by using non-enzymatic cell disruption methodologies.

TEK BOYA KULLANARAK P35S, TNOS VE PFMV HEDEFLİ ÇOKLU PZR

ÖZET

Genetik mühendisliği teknikleri ile modifiye edilen veya üretilen besin bitkileri geleneksel olarak genetiği değiştirilmiş (GD) bitkiler veya genetiği değiştirilmiş organizmalar (GDO) olarak isimlendirilir. Genetiği değiştirilmiş bitkilerin ekim alanları 1996 yılından 2012 yılına 100 kat artarak, GD bitkileri yakın tarihimizin en hızlı uyum sağlanan ürün teknolojisi haline getirmiştir. Farklı kurumsal yapıların ve araştırmacıların çalışmaları incelendiğinde genetiği değiştirilmiş organizmaların insan sağlığı ve çevre üzerine riskleri ile ilgili farklı sonuçların ortaya konulduğu görülmektedir. GD bitki ve ürün çeşitlerinin varlığının ve miktarının izlenmesi ve doğrulanması için düzenleme ihtiyacı GD bitki ve ürünlerin dünya çapında marketlerde görülmesi ile artmıştır. Bu nedenle, bitki ve bitki ürünlerinde GDO çeşitlerinin tespiti için güvenilir, hızlı ve düşük maliyetli methodların geliştirilmesine ihtiyaç vardır.

GDO analizinde ilk adım genellikle GDO' larda bulunan promotör veya terminatörler bölgelerinin taranmasıdır. GDO pozitif olarak tespit edilen örnekler üzerinde daha sonra kalitatif ve kantitatif olay spesifik ileri analizler pozitif tespitin kontaminasyondan kaynaklanmadığından emin olunması için gerçekleştirilmelidir. Bu durum GD ürünlerin etiketlenmesi için tanımlanmış eşik seviyelerinin olduğu ülkelerde de ciddi bir öneme sahiptir.

Eş zamanlı PZR GDO tespiti ve kantifikasyonu için en yaygın kullanılan tekniktir. Bu teknik ile hedef genin çoğalması, floresan boyalar kullanılarak eş zamanlı olarak görüntülenebilir. En sık kullanılan floresan boyalar oligonükleotid problar, yüksek çözünürlükte erime boyaları ve DNA bağlama boyalarıdır. En spesifik tespit sadece hedef dizilerine bağlanan oligonükleotid problar kullanılarak yapılabilir. Bu nedenle yüksek maliyetli olmalarına rağmen oligonükleotit problar en çok tercih edilen floresan boyalardır. DNA bağlama boyaları ve yüksek çözünürlükte erime boyaları çift iplikli DNA molekülüne bağlanırlar. DNA bağlama boyaları belli bir konsantrasyonun üstünde kullanıldığında PZR ‘ı inhibe edebililir. Yüksek çözünürlükte erime boyalarının minimum PZR inhibisyon etkisi vardır. Ayrıca yüksek çözünürlükte erime boyaları DNA bağlama boyaları ile karşılaştırıldığında hidrojen bağlarına 4 kat daha fazla bağlanır ve üstün erime eğrisi çözünürlüğü elde edilir.

Eş zamanlı PZR ile GDO tespitinde en çok hedeflenen diziler karnabahar mozaik virüse ait 35S promotörü (p35S); karnabahar mozaik virüse ait 35S terminatorü (t35S); figwort mozaik virüse ait 35S promotörü (FMV); Agrobacterium tumefacien’e ait nopalin sentaz geni terminatörü (tNOS), nopalin sentaz promotörü (pNOS) ve 5-enolpyruvylshikimate-3-phosphate sentaz (epsps) geni; Streptomyces

hygroscopicus'a ait bar geni (BAR); Streptomyces viridochromogenes'a ait phosphinotricin-Nacetyltransferases (pat) genleridir.

Bu çalışmada, GDO' lu bitkilerin %99 'undan fazlasının içerdiği karnabahar mozaik virüse ait 35S promotörü (p35S); figwort mozaik virüse ait 35S promotörü (pFMV); *Agrobacterium tumefacien*'e ait nopalin sentaz geni terminatörü (tNOS) dizilerinin aynı anda tespiti için tek bir yüksek çözünürlükte erime (HRM) boyası kullanılarak çoklu eş zamanlı PZR methodu geliştirildi. Farklı PZR ürünleri arasındaki ayrım hedef DNA' ların erime sıcaklıkları farklılıklarına dayalı olarak yapılmıştır. Ayrıca, bu elementlerin taranması için gerekli olan toplam analiz süresini kısaltmak için enzim içermeyen DNA ekstraksiyon yöntemi geliştirildi.

PZR tabanlı metodolojilerde hassas ve etkili sonuçlar elde etmek için yüksek kaliteli DNA gereklidir. Bu çalışmada, yüksek miktarda ve kalitede DNA elde etmek için soya ve mısır örnekleri üzerinde 5 farklı silika kolon tabanlı DNA ekstraksiyon protokolleri denenmiştir. Protokollerin üçü enzimatik adımlara dayanırken diğer 2 protokol ise tamamen kimyasal ve fiziksel hücre parçalama yöntemine dayanmaktaydı. Yöntemlerin hepsinde guanidin tiyosiyanat PZR inhibitörü inaktivasyonu ve DNA bağlanması için bir kaotropik ajan olarak kullanılmıştır. GDO tespiti için mevcut DNA ekstraksiyon metodolojileri en az 1,5 µg DNA ve 1.6 ve 2.0 arasında $A_{260/280}$ oranı ile sonuçlanmalıdır. Tüm geliştirilmiş methodlardan elde edilen DNA ekstraktların $A_{260/280}$ oranı istenilen aralıkta elde edildi. Tüm protokoller ile minimum limitden en azından 10 kat daha fazla olan 15 µg' dan daha fazla DNA elde edildi. DNA konsantrasyonu açısından en iyi sonuçları boncukla homojenizasyon ve hekzasetiltrimetil amonyum bromür (STAB) muamelesi içeren protokollerden elde edilmiştir.

Proteinler 280 nm' de absorbladığı için $A_{260/A280}$ oranı DNA ektraktlarındaki proteinlerin varlığının hesaplanmasında kullanılır. Diğer taraftan, karbonhidratlar, fenoller, aromatik bileşikler ve ağır metaller gibi diğer tip PZR inhibitörlerinin varlığı da PZR sonuçlarını etkileyebilir. Karşılaştırmalı olarak eş zamanlı PZR verimliliğine farklı protokoller ile elde edilen DNA kalitesinin etkisini değerlendirmek için her protokolden aynı miktarda DNA kullanılarak eş zamanlı PZR gerçekleştirildi. Eş zamanlı PZR çalışmalarında genel bitki kloroplast DNA' sını hedefleyen PZR primerleri kullanıldı. DNA konsantrasyonu ve saflığı farklı protokollerden elde edilen tüm seyreltilmiş DNA' lar için aynı olması nedeniyle elde edilen Ct değerleri PZR inhibitörlerinin varlığına işaret etmiştir. Tüm DNA örnekleri bitki kloroplast DNA' sına spesifik erime sıcaklığında pik vermiştir. Boncuk ile homojenizasyon ve STAB muamelesini içeren protokoller ile elde edilen DNA' lardan elde edilen eşik döngüsü değerleri diğer protokollere göre yaklaşık olarak 2 döngü daha düşük olarak bulunmuştur. Bu durum PZR inhibitörlerinin elimine edilmesinde bu iki protokolün daha başarılı olduğunu gösterdi. Bu iki protokol arasındaki fark proteinaz K muamelesi adımının dahil edilmesidir. DNA izolasyonu için gerekli olan maliyet ve toplam süreyi azal°ak amacıyla proteinaz K içermeyen protokol seçildi.

FMV, NOS, 35S pozitif referans gıda örnekleri akredite gıda kontrol laboratuarları tarafından temin edilmiştir. FMV, NOS, 35S pozitif gıda örneklerinden çıkarılan DNA' lar hedef spesifik primer çiftleri kullanılarak çoğaltıldı. Amplifikasyon döngülerinden sonra erime eğrisi analizi gerçekleştirildi ve hedeflenen PZR ürünlerinin T_m' leri hesaplandı. Hedef spesifik erime pikleri NOS spesifik reaksiyon için 73 ± 0.38˚C' de, FMV spesifik reaksiyon için 80˚C ± 0.28˚C' de, 35S spesifik

reaksiyon için 82.26 ± 0.29˚C' de ve bitki kloroplast DNA' sına specifik reaksiyon için 82 ± 0.33˚C' de elde edildi. Genellikle aynı amplikon için Evagreen kullanılarak yapılan eş zamalı PZR ile elde edilen erime sıcaklıkları 0.5 ve 1 ˚C arasında değişebileceği kabul edilmektedir. Bu çalışmada elde edilen standart sapmalar 0.4˚ C 'den daha düşük bulunmuştur. Ayrıca, bütün Evagreen eş zamanlı PZR reaksiyonları önemli ek amplifikasyon ürünleri olmadan tek bir spesifik sinyal üretmiştir.

Eş zamanlı PZR kantifikasyon standatları referans örneklerin purifiye edilmiş PZR ürünleri kullanılarak hazırlandı. Seri dilüsyonlar hedeflenen genin 10^0-10^{10} kopyasını içeren standart örnekler hazırlanması için yapıldı. Tespit limitini elde etmek için gr örnek başına 1-100 kopya 35S ve NOS içeren soya örnekleri ve gr örnek başına 1-100 kopya FMV içeren mısır örneği hazırlandı. 35S, FMV, NOS hedefli methodolojiler için tespit limiti 1 gen kopya/gr gıda örneği olarak bulunmuştur. Ancak bu çalışmada standart karışımlar referans gıda kontrol laboratuvarından elde edilmediği için methodların tespit limitleri geçek tespit limitlerinin sadece kaba tahminleridir.

35S, NOS ve FMV kalıplarının DNA karışımı primerlerin spesifikliğini test etmek için hazırlanmıştır. DNA karışımı her spesifik primer çifti ile eş zamanlı PZR ile çoğaltıldı. Eş zamanlı PZR reaksiyonlarının spesifikliği tüm amplifiye PZR ürünlerinin sekanslanması ile incelenmiştir. Sonuçlar çoğaltılmış dizilerin amaçlanan hedeflere en az %99 benzer olduğunu göstermiştir.

İlk olarak dubleks eş zamanlı PZR denemeleri her farklı DNA kalıplarının aynı miktarda eklenmesi ile gerçekleştirildi. Dubleks reaksiyonlarda daha fazla PZR ürünleri ile sonuçlanan daha fazla tercih edilen DNA kalıpları erime eğrisi analizi ile tespit edildi. FMV kalıplarından reaksiyonlarda daha fazla PZR ürünü elde edildi. 35S kalıpları ise 35S ve NOS kalıpları içeren PZR' larda daha fazla tercih edildi.

Dubleks PZR' larda farklı ilk örnek miktarının etkisini belirlemek için sadece tek bir tip T_m piki elde edene kadar çalışmalara devam edildi. Genel sonuçlar iki farklı T_m piki 1/100 rölatif kalıp konsantrasyonları altında elde edilemezken iki farklı T_m piki 1/100 rölatif kalıp konsantrasyonları üzerindeki her hedef için elde edilebildiği gösterildi. Başarılı ikili karışım denemelerinden sonra her bir referans örnekten 1000 kopya kullanılarak üçlü karışım hazırlandı. Üç primer çiftinin çoklu eşmanalı PZR' da beraber çalışabildiğini göstermek ve spesifik olmayan PZR ürünü veya primer dimeri oluşturmadığını göstermek için üçlü eş zamanlı PZR çalışmaları gerçekleştirildi. Üçlü kombinasyonlar referans örneklerin 1/1/1 röfatif kopya sayısı oranına uygulanmıştır. NOS, FMV ve 35S spesifik çoklu eş zamanlı PZR 3 farklı erime piki elde edilmesi ile sonuçlandı. NOS, FMV ve 35S hedeflerine karşılık gelen erime pikleri sırasıyla 73.04±0.13˚C, 80.21±0.10˚C ve 82.15±0.08˚C' de gözlenmiştir. Çoklu reaksiyonlarda ek amplifikasyon gözlenmemiştir.

Bitki DNA' ları her zaman GDO tarama reaksiyonlarda baskın hedef olacağından, bitki DNA' ları FMV, 35S ve NOS hedeflerinin tespit hassasiyetini artırmak için ikili ve üçlü DNA karışımlarına dahil edilmemiştir. Bitki spesifik eş zamanlı PZR' lar GDO taraması reaksiyonlarında pozitif PZR amplifikasyon kontrolü olarak kullanılmıştır.

Daha önce akredite gıda kontrol laboratuvarları tarafından analiz edilen ham ve işlenmiş gıda örnekleri geliştirilen metodoloji kullanılarak tekrar analiz edildi. Köfte, soya yağı, soya unu, mısır, mısır yağı, don yağı, kedi ve köpek mamaları, çikolata, baklava ve ekmek çeşitlerini içeren toplam 96 örnek analiz edildi. Sonuçlarımız akredite gıda kontrol laboratuvarlarında elde edilen sonuçlar ile %100 uygumludur.

Bu çalışma tek bir yüksek çözünürlükte erime boyası kullanılarak 35S, NOS ve FMV bölgelerinin eş zamanlı çoklu tespitinin mümkün olduğunu göstermiştir. Ayrıca enzimatik olmayan hücre parçalama yöntemlerini kullanarak yüksek kalitede DNA elde edilmesinin mümkün olduğu gösterilmiştir.

1. INTRODUCTION

Genetically modified organisms can be defined as the organisms into which one or several genes coding for desirable traits have been inserted by the process of genetic engineering. These genes may stem not only from the same or other plant species, but also from organisms totally unrelated to the recipient crop. 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 (Tung et al., 2009).

Food plants that are being produced or modified by genetic engineering techniques are named in literature as genetically engineered plants, bio-engineered plants, genetically modified organisms, genetically modified (GM) crops, or biotech plants (Liu, 1999; Wilkinson, 1997).

Investigations of different industrial centers and researchers reveal different results involved in the risks of GMOs on human health and the environment (Seralini, 2012; Chelsea, 2012). The regulatory need to monitor and verify the presence and the amount of GM varieties in crops and products has increased with the release of GM crops and products in the markets worldwide. Labeling legislation and trade requirements differ from one country to another. Therefore, there is need to determine whether only officially approved transgenic events used commercially in the country. Consequently, reliable and sensitive methods need to be developed for the detection of GM varieties in crops and their products.

1.1 Purpose of the Thesis

In this study, we developed a multiplex QPCR methodology using a single high resolution melting dye to simultaneously detect Cauliflower Mosaic Virus 35S promoter, *Agrobacterium tumefaciens* Nopalin Synthase terminator and Figworth mosaic virus 35S promoter, which are contained in more than 99% of the GMO events (Oliver, 2012). Discrimination between the different PCR products was based on the differences in melting temperatures of the target DNAs. We also developed an

enzyme free DNA extraction methodology for food samples to shorten the total analysis time necessary for the screening of these elements.

2. GENETICALLY ENGINEERED CROPS

2.1 Current Status of Commercial GM Crop Production

In 2012, the global area of biotech crops continued to increase for the 17th year at a sustained growth rate of 6% or 10 million hectares (25 million acres), reaching 170.3 million hectares or 420 million acres (Table 2.1). Biotech crops have set a precedent in that the biotech area has grown impressively every single year for the past 17 years, with almost a remarkable 100-fold increase since the commercialization began in 1996. Thus, biotech crops are considered as the fastest adopted crop technology in the history of modern agriculture (James, 2012).

Table 2.1 : Global area of biotech crops, 1996 to 2012 (James, 2012).

	Hectares (Million)	**Acres (Million)**
1996	1.7	4.3
1997	11.0	27.5
1998	27.8	69.5
1999	39.9	98.6
2000	44.2	109.2
2001	52.6	130.0
2002	58.7	145.0
2003	67.7	167.2
2004	81.0	200.0
2005	90.0	222.0
2006	102.0	250.0
2007	114.3	282.0
2008	125.0	308.8
2009	134.0	335.0
2010	148.0	365.0
2011	160.0	395.0
2012	170.3	420.8
TOTAL	**1,427.3**	**3,531.8**

Biotech crops were grown commercially in all six continents of the world. In 2012, 28 countries were planting biotech crops of which 20 developing countries and 8 industrial ones. Between the developing countries, Brazil is the leader with 30.3 million hectares and an increase of 20% was seen compared to 2011. The other main countries are Argentina (23.7 million ha), India (10.6 million ha cotton), China (3.9 million ha), and South Africa (2.3 million ha). The United States of America are still

the lead producer of biotech crops amongst the industrial countries with 69 million hectares of biotech crops and an increase of 5% in the last year. 17 countries planted 50,000 hectares or more to biotech crops (Table 2.2). These mega-countries included the UUSA, Brazil, Argentina, Canada, India, China, Paraguay, South Africa, Pakistan, Uruguay, Bolivia, Philippines, Australia, Burkina Faso, Myanmar, Mexico, Spain, and Chile (Broeders, 2012; James, 2012).

Table 2.2 : Global area of biotech crops by country (James, 2012).

Country	2011 (Million hectares)	2012 (Million hectares)
USA	69.0	69.5
Brazil	30.3	36.6
Argentina	23.7	23.9
Canada	10.4	11.6
India	10.6	10.8
China	3.9	4.0
Paraguay	2.8	3.4
South Africa	2.3	2.9
Pakistan	2.6	2.8
Uruguay	1.3	1.4
Bolivia	0.9	1.0
Philippines	0.6	0.8
Australia	0.7	0.7
Burkina Faso	0.3	0.3
Myanmar	0.3	0.3
Mexico	0.2	0.2
Spain	0.1	0.1
Chile	<0.1	<0.1
Colombia	<0.1	<0.1
Honduras	<0.1	<0.1
Sudan	<0.1	<0.1
Portugal	<0.1	<0.1
Czech Republic	<0.1	<0.1
Cuba	<0.1	<0.1
Egypt	<0.1	<0.1
Costa Rica	<0.1	<0.1
Romania	<0.1	<0.1
Slovakia	<0.1	<0.1

It is currently estimated that biotech soybean continued to be the principal biotech crop in 2012, occupying 80.7 million hectares or 47% of global biotech area. İt is followed by biotech maize (55.1 million hectares at 35%), biotech cotton (30 million hectares at 15%) and biotech canola (9.2 million hectares at 3%) of the global biotech crop area (Figure 2.1) (James, 2011).

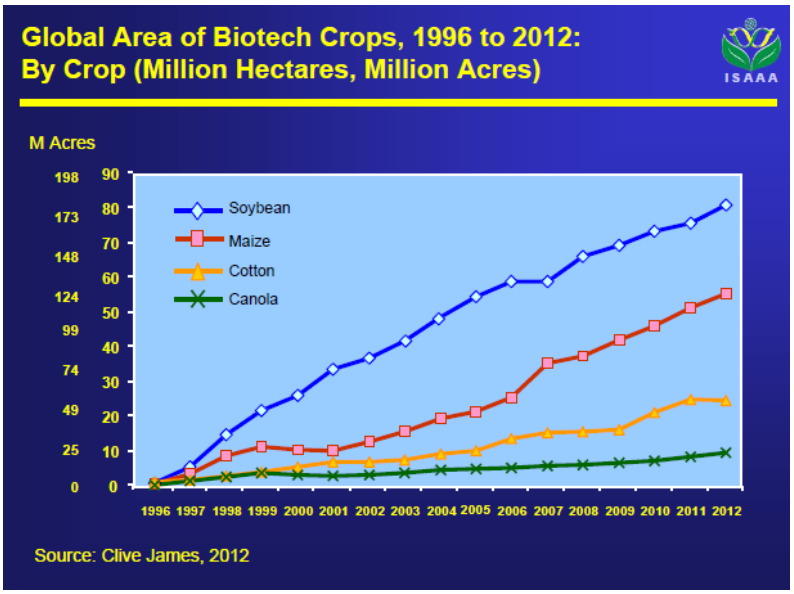


Figure 2.1 : Global area of biotech crops, 1996 to 2012: by crop (James, 2012).

Herbicide tolerance, insect resistance and a combination of these are most widely adopted GM traits. Table 2.3 shows distribution of transgenic crops by trait. Herbicide tolerant soybean continued to be the dominant biotech crop in 2012, occupying 80.7 million hectares or 47% of global biotech area. The second most dominant crop was biotech maize with stacked traits, which occupied 39.9 million hectares or 23% of the global biotech area. Biotech (Bt) cotton was the third most dominant crop grown in 2012. Bt cotton was planted in more than 18.8 million hectares (11% of the global biotech area) (James, 2012).

Table 2.3 : Dominant biotech crops in 2012 (James, 2012).

Crop	Million Hectares	% Biotech
Herbicide tolerant soybean	80.7	47
Stacked traits maize	39.9	23
Bt cotton	18.8	11
Herbicide tolerant canola	9.2	5
Herbicide tolerant maize	7.8	5
Bt maize	7.5	4
Stacked traits cotton	3.7	2
Herbicide tolerant cotton	1.8	1
Herbicide tolerant sugar beet	0.5	<1
Herbicide tolerant alfalfa	0.4	<1
Others	<0.1	<1
Total	170.3	100

2.2 Application of GM Technology in Crops

The world population is expected to increase from the current 6.7 billion to 9 billion by 2050. On the other hand, the world's arable land will not increase and most likely it will reduce due to industrial development and urbanization in developing countries. It was estimated that a 50 percent increase in food demand alone is required by 2030 (Royal Society, 2009). GM foods are important tools to find a solution of this problem.

Three categories of GM traits can be distinguished. The first generation of GM crops contains improvements in agronomic traits, such as better resistance to pests and diseases and tolerance to herbicides. GM crops tolerant to abiotic stress such as drought, heat, and salt is also being worked on intensively. The second generation of GM crops involves product quality improvements for nutrition and industrial purposes. Examples include canola, cotton, linseed, maize, palm, peanut, rice, soybean, safflower and Sunflower with improved fatty acid profiles; maize with enhanced amylose content; staple foods with enhanced contents of essential amino acids, minerals, and vitamins; and GM functional foods with diverse health benefits. The third generation of crops are plants designed to produce special substances for pharmaceutical or industrial purposes. Types of genetic transformations are given below.

2.2.1 Herbicide tolerant crops

Weeds have adverse effects such as competition with nutrients and sunlight on production of plant crops. Herbicides spray used to control weeds over the past 50 years. However, many of the herbicides are toxic or slightly toxic to animals and humans. The Environmental Protection Agency (EPA) uses four toxicity classes. Classes I and II are toxic and moderately toxic. Class III is slightly toxic and class IV is practically nontoxic. Some newer herbicides like the herbicide glyphosate (trade name Roundup) are considered nontoxic (class IV). It is essentially a modified amino acid that blocks a chloroplast enzyme called 5-enolpyruvoyl-shikimate-3-phosphate synthetase (EPSPS). Glyphosate binds more tightly to the EPSPS-shikimate-3'phosphate complex than does phosphoenolpyruvate (PEP). Consequently, EPSPS is effectively inactivated once glyphosate binds to enzyme-substrate complex.

EPSPS is a key enzyme in the biosynthesis of aromatic amino acids phenylalanine, tyrosine and tryptophan that is required for plant, but not animal (Figure 2.2).

In herbicide tolerant crops, a glyphosate-tolerant EPSPS gene derived from the bacterium *Agrobacterium tumefaciens* is engineered into the plant. Growers of herbicide-tolerant crops can spray glyphosate to control weeds without harming their crop when gene expressed in GM plants (Pamela Ronald, 2011). EPSPS is the only physiological target of glyphosate in plants, and no other PEP-utilising enzymes are inhibited by glyphosate.

Roundup Ready soybean (GTS 40-3-2) is engineered to tolerant herbicide Glyphosate. The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene is under the regulation of a strong constitutive promoter from Cauliflower Mosaic Virus (E35Sp) and terminates with the nopaline synthase terminator (tNOS) derived from *Agrobacterium tumefaciens* (Figure 2.3) (Querci et al., 2006).

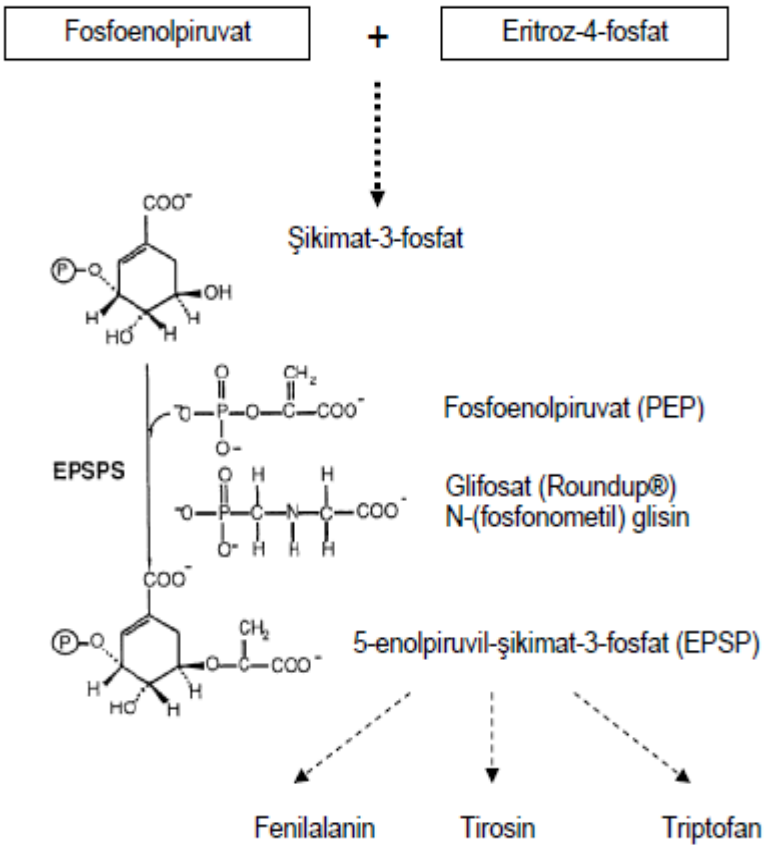


Figure 2.2 : EPSPS catalyses the reaction of shikimate-3-phosphate and PEP to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and phosphate (Querci et al., 2006).

Figure 2.3 : Schematic representation of the Roundup Ready soybean gene cassette (Querci et al., 2006).

The development of GTS 40-3-2 was based on recombinant DNA technology, through the introduction of a glyphosate tolerant form of the enzyme EPSPS gene, isolated from *Agrobacterium tumefaciens* strain CP4, into the commercial soybean variety "A5403" (Asgrow Seed Company) by particle bombardment. A plant-derived DNA sequence coding for a chloroplast transit peptide (CTP4 from Petunia hibrida) was cloned at the 5' of the glyphosate tolerance gene. The signal peptide fused to the EPSPS gene facilitates the import of newly translated enzyme into the chloroplasts, where both the shikimate pathway and glyphosate sites of action are located. Once importation has occurred, the transit peptide is removed and rapidly degraded by a specific protease (GM crop database).

The commercial soybean variety A5403 (Asgrow Seed Co.) was transformed by means of gold particle bombardment; with the PV-GMGT04 plasmid vector harvested from *Escherichia coli* (Figure 2.4). The PV-GMGT04 plasmid contained the CP4 EPSPS gene coding for glyphosate tolerance, the gus gene for production of ß- glucuronidase as a selectable marker, and the npt II gene for antibiotic resistance (kanamycin). Roundup Ready® (RR) soybean is, at present, the only transgenic soybean line approved for marketing in the EU, after clearance in the US in 1994.

2.2.2 Insect resistant crops

Insect resistant crops are engineered to produce a toxin protein from the soil bacteria *Bacillus thuringiensis* (Bt) in most of their tissues. Bt toxins cause no harm to most non-target organisms including beneficial insects, wildlife, and people. Whereas they cause death including susceptible insects when they eat Bt crops (Pamela Ronald, 2011).

The genes encoding hundreds of Bt toxins have been sequenced. Most of the Bt toxins used in transgenic crops are called Cry toxins because they occur as crystalline

proteins in nature (Crickmore et al., 2011). Strains of *B. thuringiensis* produce a wide range of different crystal proteins (Table 2.4) (Slater et al., 2003).

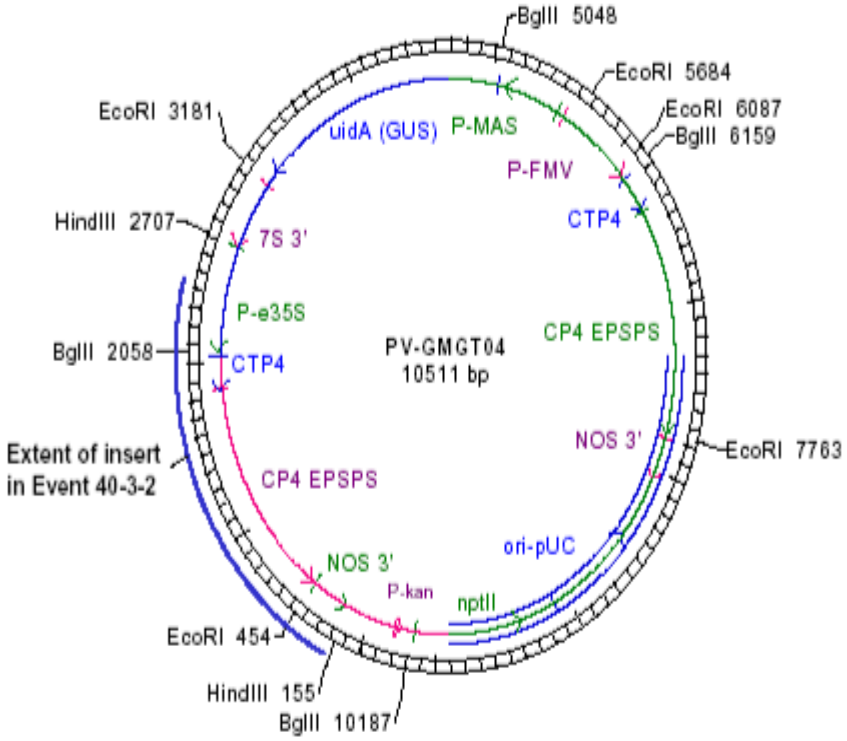


Figure 2.4 : Plasmid map including genetic elements of vector PV-GMGT04 used in the transformation of RR soybean event 40-3-2 (Querci et al., 2006).

The mode of action of endotoxins involves a specific interaction between the protein and the insect larva midgut. After ingestion by an insect larva, the protein crystals are solubilized in its midgut. The larger protein such as the 130 kDa Cry1 group are proteolytically cleaved at this stage to release active 55-70 kDa active fragment of the protein. This interacts with high affinity receptors in the midgut brush-border membrane. The result of this binding is to open cation-selective pores in the membrane. The flow of cations into the cells results in osmatic lysis of the midgut epithelium cells, causing their destruction. Thus, the endotoxins are extremely toxic and can be lethal to susceptible insect larvae at relatively low concentrations. The conditions in the insect larva midgut vary according to insect class. The midgut of Lepidoptera and Diptera is midly alkaline, whilst the coleopteran gut is generally either more alkaline or acidic. These different conditions favour the solubilisation and activation of different Cry subfamilies. In addition, the specificity of the interaction between the endotoxin and the midgut receptor means that individual Cry proteins are active against particular insect larvae (Slater et al., 2003).

Table 2.4 : The range of insecticidal cry proteins in individual *Bacillus thuringiensis* strains (Slater et al., 2003).

B.t. Subpecies and strains	**Crystal protein**
Aizawai	Cry1Aa, Cry1 Ab, Cry1Ad, Cry1Ca, Cry1Da, Cry1Eb, Cry1Fa, Cry9Ea, Cry39Aa, Cry40Aa
Entomocidus	Cry1Aa, Cry1Ba, Cry1Ca, Cry1Ib
Galleriae	CryAb, CryAc, Cry1Da, Cry1Cb, Cry7Aa, Cry8Da,Cry9Aa, Cry9Ba
Israelensis	Cry10Aa, Cry11Aa
Japonensis	Cry8Ca, Cry9Da
Jegathesan	Cry11Ba, Cry19Aa, Cry24Aa, Cry25Aa
Kenyae	Cry2Aa, Cry1Ea, Cry1Ac
Kumamotoensis	Cry7Ab, Cry8Aa, Cry8Ba
Kustaki HD-1	Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ia, Cry2Aa, Cry2Ab
Kusrstaki HD-73	Cry1Ac
Kurstaki NRD-12	Cry1Aa, Cry1Ab, Cry1Ac
Morrisoni	Cry1Bc, Cry1Fb, Cry1Hb, Cry1Ka, Cry3Aa
Tenebrionis	Cry3Aa
Tolworthi	Cry3Ba, Cry9Ca
Wuhanensis	CryBd, Cry1Ga, Cry1Gb

Maize event MON810 (YieldGard®) was developed by Monsanto Canada Inc. to be specifically resistant to European Corn Borer (ECB; Ostrinia nubilalis). MON810 was developed using recombinant DNA technology and micro projectile bombardment of plant cells, to introduce a gene encoding the production of a naturally occurring insecticidal protein (derived from *Bacillus thuringiensis ssp. kurstaki*). This protein is active against certain species of Lepidoptera, the insect order to which butterflies and moths belong, including ECB.

More specifically, the protein expressed in MON810 is a truncated form of the insecticidal protein, CRYIA(b) toxin. It was modified to optimize and maximize the expression of the toxin CRYIA(b) protein in plants. The native protein has a molecular weight of 131 kD. The inserted, plant expressed cryIA(b) gene codes for a truncated protein with a molecular weight of 91 kD. After activation by trypsin to the insecticidal form, the resulting proteolytic fragments were compared to the bacterial

proteins and shown to be of similar molecular weight, amino acid sequence, and immunological reactivity.

MON810 was obtained from maize genotype Hi-II by biolistic transformation with a mixture of plasmid DNAs, PV-ZMBK07 (Figure 2.5) and PV-ZMGT10. The PV-ZMBK07 plasmid contained the enhanced cauliflower mosaic virus 35S promoter, the maize hsp70 intron 1 and the synthetic toxin cryIA(b) gene followed by the NOS terminator (Figure 2.6). PV-ZMGT10 plasmid contained the CP4 EPSPS and glyphosate oxidoreductase (gox) genes. Gox genes degrade glyphosate in to a non toxic compound. Both plasmids also contained the nptII gene (for bacterial selection) under the control of a bacterial promoter, and an origin of replication from a pUC plasmid (ori-pUC) required for replication of the plasmids in E. coli. The two vectors were introduced by micro projectile bombardment into cultured plant cells (Querci et al., 2006). Glyphosate tolerant transformed cells were selected and subsequently cultured in tissue culture medium for plant regeneration (Armstrong et al., 1991).

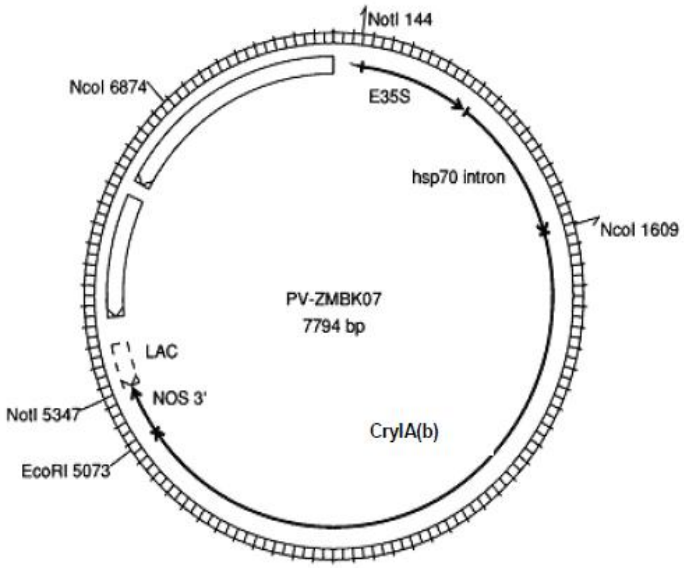


Figure 2.5 : Schematic representation of the plasmid PV-ZMBK07 used in engineering MON810 (Querci et al., 2006).

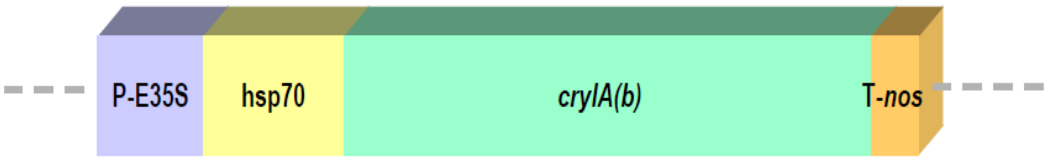


Figure 2.6 : Schematic representation of the cryIA(b) construct from plasmid PV-ZMBK07 used in the transformation of MON810 (Querci et al., 2006).

Molecular analyses provided by the authors indicated that only the elements from construct PV-ZMBK07 were integrated into the genome of line MON810 as a single insert, consisting of the enhanced 35S promoter, the hsp70 leader sequence and the truncated cryIA(b) gene (BATS, 2003). The CP4 EPSPS and gox protein encoding genes were presumed to have been inserted into the initial transformant at a separate genetic loci from the cry1Ab gene and then subsequently lost through segregation during the crossing events leading to line MON810 (Querci et al., 2006).

2.2.3 Disease resistant crops

Plants can be genetically modified to be resistant to bacterial, fungal or viral infestation. A transgene makes crops resistant to biotic stresses such as plant pathogens which often reduce yields substantially. Examples of crops in which these traits are being introduced include coffee, bananas, cassava, potato, sweet potato, beans, wheat, papaya, squash and melon.

The first and most successful approach to viral resistance has been with the transgenic expression of the coat protein (CP) coding sequence. CP mediated resistance was first reported with a TMV-tobacco model system in 1986. Subsequently, a large number of transgenic lines containing CP transgenes have been produced for a whole range of crop species and many different viruses (Slater et al., 2003).

In 1998, Papaya lines 55-1 and 63-1 were engineered for infection resistance by papaya ring spot virus (PRSV), a major limiting factor in papaya production. Virus-derived sequences encoding the PRSV coat protein were inserted in this papaya lines. The introduced viral sequences do not result in the formation of any infectious particles and enables the plants to resist infection against PRV.

The transgenic papaya lines 55-1 and 63-1 were produced by particle bombardment transformation of embryogenic cultures of the papaya cultivar Sunset. The *Agrobacterium tumefaciens* binary plasmid pGA482GG/cpPRSV-4 used for the transformation contained three plant-expressible genes, the PRSV CP, *neo*, and *gus* genes. neo and gus genes serve as genetic marker genes. The plasmid also had two genes encoding resistance to tetracycline and gentamycin antibiotics, respectively, but their associated DNA regulatory sequences enabled expression only in bacteria.

The plasmid included the right- and left-border regions derived from the *A. tumefaciens* T-DNA (GM crop database).

Expression of the PRSV CP gene was controlled by including promoter and transcription termination and polyadenylation signal sequences derived from the 35S transcript of cauliflower mosaic virus (CaMV). In addition, the CP gene sequences were fused to the 5' untranslated sequence and the first 39 nucleotides from the cucumber mosaic virus (CMV) CP to enhance translation of the transgene mRNA. The inclusion of these additional sequences was necessary because PRSV naturally encodes its CP as part of a polyprotein and, therefore, the CP coding region normally lacks a translation initiation ATG codon (Figure 2.7).

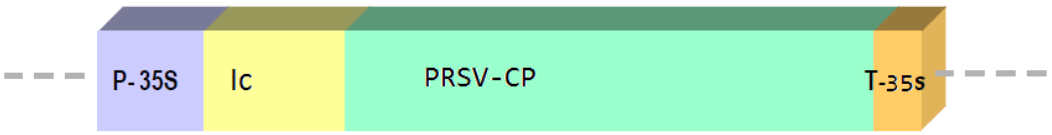


Figure 2.7 : Schematic diagrams of the constructs for transgenic papaya. P- 35S CaMV 35S promoter, Ic-5′untranslated region and translation initiation codon from cucumber mosaic virus (CMV), PRSV-CP papaya ringspot virus coat protein gene, T-35S CaMV 35S terminator (Querci et al., 2006).

2.2.4 Transformation for nutritional purposes and pharmaceutical purposes

Golden rice, an example of transgenic crops for nutritional purposes, has been discussed as a possible cure for Vitamin A deficiency. Vitamin A deficiency is estimated to result in 2 million people becoming blind each year especially in Africa and Southeast Asia. To combat Vitamin A deficiency, GM rice has been developed with increased beta-carotene (precursor of vitamin A) content and resulting genetically engineered plants were named “Golden Rice.”

Figure 2.8 shows biosynthetic pathway of provitamin A. The addition of isopentenly diphoshate (IPP), and dimethylallyl diphosphate (DMAPP) to 20-carbon (geranyl geranly diphosphate (GGPP)) is an important starting point for the synthesis β-carotene. GGPP is then converted to β-carotene by phytoene desaturase and ζ-carotene desaturase and lycopene β-cyclase. Immature rice endosperm is capable of synthesising GGPP, but subsequent stages of the pathway are not expressed in this tissue. Early transformation experiments with a phytoene synthase (psy) gene from daffodil fused to a rice endosperm-specific promoter indicated that phytoene could

be synthesised from GGPP in the rice grain. However, three subsequent steps are required to convert phtoene to β-carotene; phytoene desaturase and ζ-carotene desaturase to introduce the double bonds to form lycopene, and lycopene β-cyclase to form the rings in β-carotene. Fortunately, a bacterial carotene desaturase gene capable of introducing all four double bonds can be substituted for the phytoene desaturase and ζ-carotene desaturase (Figure 2.8). Nevertheless, the manipulation of Golden Rice requires the introduction of three genes: phytoene synthase, carotene desaturase and lycopene β-cyclase.

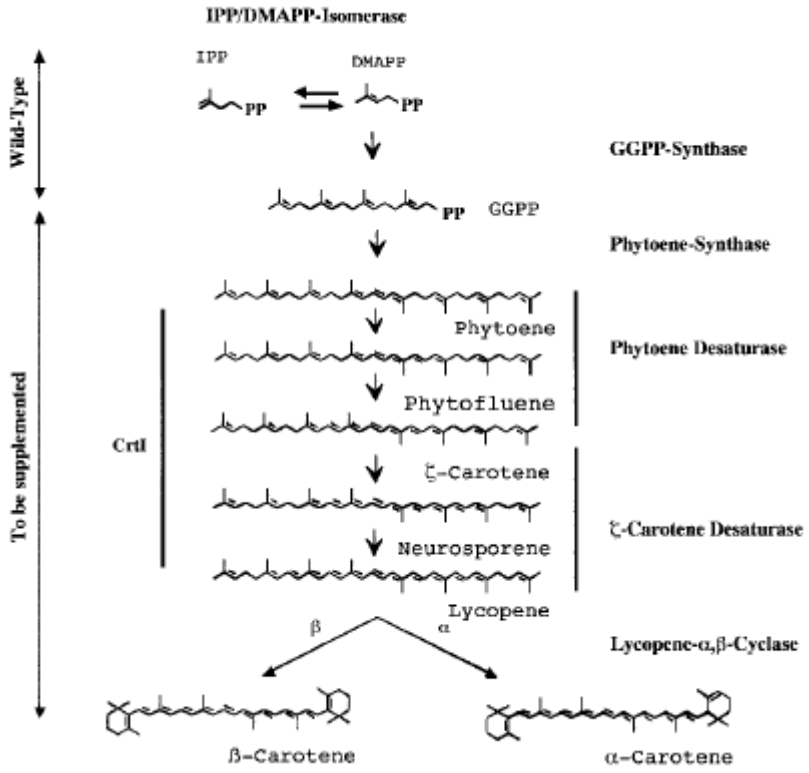


Figure 2.8 : Provitamin A biosynthetic pathway (Beyer et al., 2002).

The constructs used to target expression of the appropriate genes to the rice endosperm are shown in Figure 2.9. The most successful strategy for the production of Goden Rice involved transformation with two independent constructs. The first one that contains a daffodil phytoene synthase (psy) gene fused to a rice glutelin promoter (Gtl P) along with a bacterial carotene desaturase gene (ctr 1) from Erwinia uredovora controlled by the 35S promoter inserted into the vector pZPsC. Both enzymes were targeted to the plastid (the site of GGPP synthesis): the psy gene by its own transit peptide, and the ctr 1 gene by fusion to a pea rbcS (ribulose-1,5-

bisphosphate carboxylase/oxygenase small subunit) transit peptide sequence. The second construct that contains the lycopene b-cyclase (lcy) gene from daffodil with a functional transit peptide was inserted into the vector pZLcyH under the control of the rice endosperm-specific glutelin promoter, along with a hygromycin-resistance selectable marker gene (aph IV) (GM crop data base).

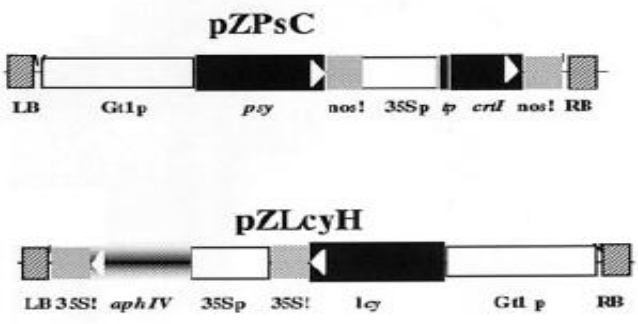


Figure 2.9 : Constructs for the production of Golden Rice (Beyer et al., 2002).

The first version of Golden Rice was criticized because it contained too little beta-carotene (a maximum of 1.6 µg/g) to be effective (Ye et al., 2000). Subsequently the second generation of Golden Rice (Golden Rice II) was developed with improved carotene production ranging from 9 to 37µg/g (Paine et al., 2005). According to the update report of the International Rice Research Institute (IRRI), Golden Rice is still under development and evaluation.

The advantages of edible vaccines for viral and diarrheal diseases using proteins expressed in transgenic plants would be enormous, especially for developing countries. Bananas that produce human vaccines against infectious diseases such as Hepatitis B have been developed but are not in production (Kumar 2005). Tobacco plants that can produce therapeutic antibodies have been developed and studied, but they are not in production (Jha et al., 2012).

2.2.5 Transformation with desirable quality genes

The development of GM crops with desirable quality is also predicted to be broadly beneficial. Plants engineered to tolerate abiotic stresses like drought, frost and nitrogen starvation were in development in 2013.

The first GM crop with enhanced quality was the FlavrSavr tomato, which had a longer shelf life. Flavr savr tomatoes were developed using recombinant DNA techniques to express the trait of delayed softening of tomato fruit.

Fruit ripening is an active process that, in climacteric fruit such as tomatoes, is characterized by a brust of respiration, ethylene production softening and changes to colour and flavor. Ethylene production is significant, because ethylene is known to be the phytohormone that triggers ripening in climacteric fruit. The softening of the fruit is largely the results of the cell wall degrading activity of the enzymes polygalacturonase (PG) and pectin methylesterase (PME). The PG enzyme is synthesized de novo during ripening and acts to break down the polygalacturonic acid chains that form pectin glue of the middle lamella, which stricks neighbouring cells together (Slater et al., 2003).

The Flavr savr tomato was developed by antisense concept. The basic antisense concept involves creating a construct in which the gene sequence is transcribed in the reverse orientation, using opposite strand as the template. Therefore the resulting antisense transcript has a sequence complementary to the normal (sense) mRNA. Some interactions at the transcriptional, post transcriptional or translational level would reduce the expression of the endogenous mRNA. This turned out to be the case indicated that the levels of both sense and antisense RNA were reduced. The mode of action is that ds RNA hybrids formed between the antisense RNA and endogenous mRNA are recognised by plant cell-defense mechanisms and degraded (Slater et al., 2003).

The Flavr savr tomato was developed by insertion of an additional copy of the polygalacturonase encoding gene in the antisense orientation, resulting in reduced translation of the endogenous PG messenger RNA (mRNA). Reduced PG expression decreases the breakdown of pectin and leads to fruit with slowed cell wall breakdown, better viscosity characteristics and delayed softening.

This bioengineered tomato was produced by *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum*) in which the transfer-DNA (T-DNA) region of the bacterial tumour inducing (Ti) plasmid was modified to contain DNA sequences encoding an antisense PG gene construct and the nptII encoding *neo* gene from *E. coli* K12.

The antisense PG gene was under the regulatory control of a single copy of the 35S promoter from the cauliflower mosaic virus (CaMV), or two tandem copies of the 35S promoter. The terminator sequences were from the *tml (tumor morphology lerge gene)* gene from *A. tumefaciens*. Expression of the *neo* gene was under the control of the 5' promoter and 3' terminator sequences from the mannopine synthase gene derived from *A. Tumefaciens* (Figure 2.10) (GM crop database).

Flavr Savr (also known as CGN-89564-2) was the first commercially grown genetically engineered food to be granted a license for human consumption. It was produced by the Californian company Calgene, and submitted to the U.S. Food and Drug Administration (FDA) in 1992 (Redenbaugh et al., 1992). It was first sold in 1994, and was only available for a few years before production ceased in 1997. Currently there are no genetically modified tomatoes available commercially.

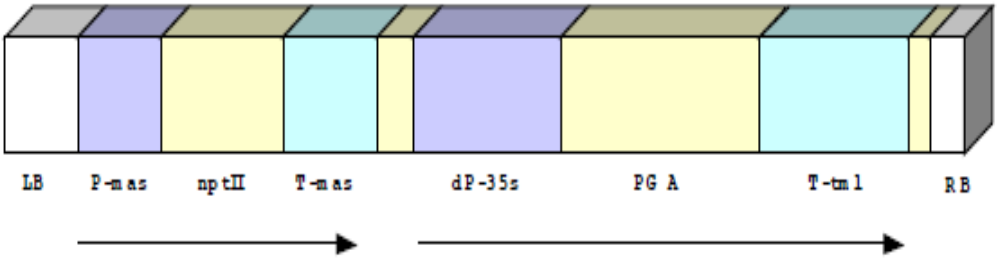


Figure 2.10 : Construct for the production of Flavr savr tomato (BATS report. 2003).

In 2012, an apple has been genetically modified to resist browning in Canada. A gene has been modified to produces less polyphenol oxidase in the fruit.

2.4 Methods of Gene Transfer in Plants

The most commonly used methods to transform a plant are *Agrobacterium tumefaciens* method and direct DNA transfer methods. *A. tumefaciens* can transfer a particular DNA segment named Transfer DNA (T-DNA) of the tumor inducing (Ti) plasmid into the host genome and causes crown gall disease in a wide range of plants (Alimohammadi, 2009). The foreign gene that cloned in the T-DNA region of Ti-plasmid in place of unwanted sequences can be transferred and integrated into plant genome (Querchi, 2006).

A. tumefaciens naturally infects only dicotyledonous plants. Therefore, genetic manipulation of many important plants remains accessible only by other methods such as chemical procedures (polyethyleneglycol-mediated transfer), electroporation and microparticle bombardment (gene gun, biolistic) technology. But recent studies have shown that Agrobacterium-mediated gene transfer methods can be also used in transformation of monocotyledonous like rice, banana, corn and wheat (Babu et al., 2003).

Direct DNA transfer methods are useful for both stable transformation and transient gene expression. For direct DNA transfer methods protoplasts are ideal to gene transfer. DNA can be introduced into plant protoplasts via polyethylene glycol (PEG) fusion, electroporation. Protoplasts treated with chemicals like polyethylene glycol more readily take up DNA from their surrounding medium. Electroporation involves short high-voltage electrical pulses applied to protoplasts to induce transient pores in the plasma membrane and this facilitates the uptake of DNA. A major disadvantage of methods utilizing protoplasts is that the regeneration of plants from protoplast cultures can be a complex and time-consuming process and the frequency of stable transformation is low (Newell, 2000).

Other method used to transfer foreign DNA into plant cells is Particle bombardment (Yao et al., 2006). Gold or tungsten particles (1–2 μm) coated with the DNA are loaded into a particle gun and accelerated to high speed and bombarded onto the target tissue or cells using a particle gun.

2.5 Genetic Elements Used in Transgenic Crops

The genes that encode the traits of interest can be inserted into the plant genome using transformation. Currently genetically modified plants are mainly transformed using a transgenic insert (gene cassette). This gene cassette contains a promoter region, a coding sequence (trait), and a terminator. A promoter region at the upstream side of the coding sequence of the gene provides a correct expression in the plant. A terminator region at the end of the coding region of the gene provides transcription termination and polyadenylation (BATS report, 2003).

Mostly the cauliflower mosaic virus cauliflower 35S promoter and the *Agrobacterium tumefaciens* nopaline synthase terminator were used as a promoter

and terminator sequence in the first GM crops. The traits were also limited to genes conferring herbicide tolerance (HT) and insect resistance (IR). Additionally these traits were introduced into few commodity crops such as maize, soybean, and oilseed rape. The main HT sequences are the bacterial phosphinotricin-Nacetyltransferases from *Streptomyces viridochromogenes* (pat) and from *Streptomyces hygroscopicus* (bar) and the 5-enolpyruvylshikimate-3-phosphate synthase (epsps) from the *Agrobacterium tumefaciens* strain CP4 or from plant origin (in casu petunia). For the IR trait, artificial versions of the *Bacillus thuringiensis* (Bt) δ-endotoxin encoding genes (e.g., the cryIAb/Ac) have been utilized (Broeders, 2012).

In more recent years, new regulatory sequences have been introduced such as the cauliflower mosaic virus 35S terminator (t35S), the figworth mosaic virus promoter (pFMV), the *Agrobacterium tumefaciens* nopaline synthase promoter (pNOS), the rice actin promoter (pAct), and the maize ubiquitine promoter (pUbiZM). Furthermore, new genes from the Bt δ-endotoxin family are also being used now (cry3Bb, cry3A, cry1F, etc.). Moreover, more species like rice, cotton, sugarbeet, and potato are currently used for transformation (Broeders, 2012).

3. STATE –OF –THE -ART METHODS IN GM DETECTION

In response to consumer pressure, many countries have introduced labeling regulations for GM foods. Although GMO labeling does not have any bearing on the safety aspect of GMOs, it is used to give consumers a choice, between GM and non-GM, allowing them to balance concerns of morality and perceived risk (Viljoen, 2005). GM crops and their products can be identified by detecting either the inserted genetic material at DNA level or the resulting protein.

However, DNA based technologies have some advantages over protein based methods like sensitivity and specificity. Additionally, because of the protein denaturation and degradation during processing of foods, protein based methods cannot be used for the detection of GMOs in the case of processed foods. Therefore, DNA based methods can only be used for processed foods (Kim,HY. 2010).

3.1 DNA Based Detection Methods

The commonly used DNA based GMO detection techniques are southern blot, Polymerase chain reaction (PCR), and microarray analysis.

The microarray (DNA chip or biochip) consist of oligonucleotide probes attached in array format to a solid surface. These oligonucleotide probes bind to fluorescently labeled target sequences (DNA or RNA). The microarray is scanned for detection of probe-target hybridization by computer.

Recently detection of GM maize, canola, cotton and soybean events is achieved by microarrays combined with multiplex PCR methods (Leimanis et al., 2006; Xu et al,. 2007; Kim, JH. et al., 2010). Moreover, a novel multiplex quantitative DNA-based target amplification method suitable for use in combination with microarray detection (NAIMA) has been reported (Morisset et al., 2008). This fast and simple integrated method allows sensitive, specific and fully quantitative on-chip GMO detection in a multiplex format. The disadvantage of the microarray analysis is its

relatively expensive cost. İt is also considered as a one of the most promising discrimination platforms at present for GMO detection.

PCR is the most commonly used method for GMO detection and traceability among the other methods, because of its rapid and relatively low-cost detection procedures.

3.1.1 Polymerase chain reaction

The PCR technique is based on million or billion fold amplification of a specific target DNA fragment by two synthetic oligonucleotide primers. The method consists of consecutive cycles of three different temperatures. In each cycle the three temperatures correspond to three different steps in the reaction. The first step in a cycle involves separation of the two strands of the template DNA molecule into single strands by heat denaturation at ~94 °C. The second step involves cooling down reaction temperature to 50-65 °C (depending on the GC-content) and then binding of the two primers to the target sequence. Primer hybridization is favored over DNA-DNA hybridization because of the excess of primers molecules. The third step involves making two perfect copies of the original double stranded DNA molecule by a Thermus aquaticus (Taq) polymerase at the optimum temperature of 72 °C. Once the cycle is completed, it is then repeated 20 to 50 times, depending on the amount of DNA present and the length of the amplicon. The number of target sequences grows exponentially according to the number of cycles in consecutive reaction cycles (Tripathi, 2005; Anklam, 2002).

General PCR and real-time PCR systems are used as qualitative and quantitative assays for analysis of GMOs, respectively. For any PCR-based detection strategy, it is very important to know a detailed knowledge of the transgenic DNA sequences and of the molecular structure of the GMOs in order to select the appropriate oligonucleotide primers. Several parameters including the length of the primer, %GC content and the 3' sequence of primer need to be optimized for successful PCR (Anklam, 2002).

3.1.1.1 Qualitative PCR methods for GMO detection

PCR based GMO assays can be classified into at least four categories according to their level of specificity criterion (Figure 3.1). These are screening PCR, gene-specific PCR, construct-specific PCR and event-specific PCR. In each assays,

different region of DNA construct is amplified. Therefore, the choice of target sequence is the single most important factor controlling the specificity of the PCR assays. The target sequence is normally a part of the modified gene construct, for example a promoter, a terminator, a gene, or a junction between two of these elements (Holst-Jensen, 2003).

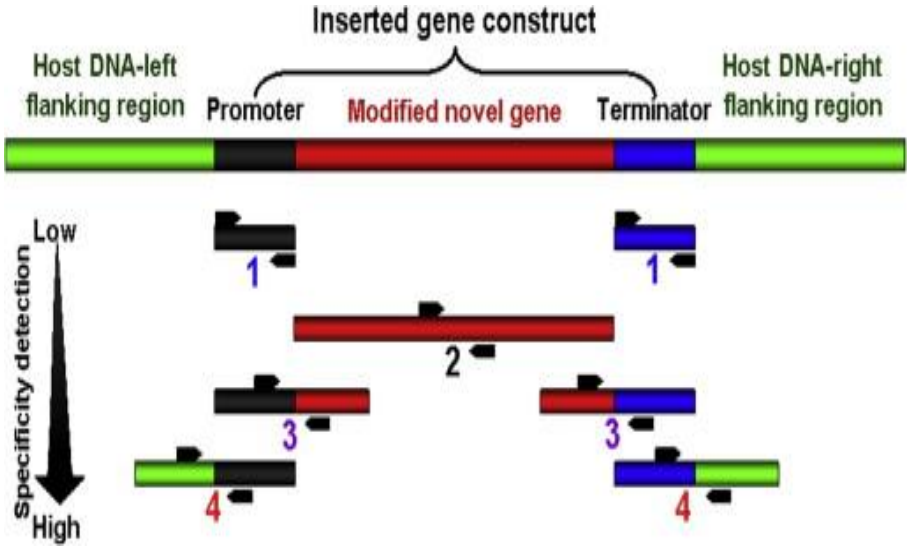


Figure 3.1 : Four main PCR strategies including screening (1), gene specific (2), construct specific (3) and event specific (4) that used in GM crops detection (Shrestha, 2010).

PCR assays can be followed by confirmation methods in order to ensure that the amplified DNA product actually corresponds to the chosen target sequence and is not a product of non-specific binding of the primers. Gel electrophoresis is the simplest method to control whether the PCR products have the expected size. However, it cannot discriminate the presence of unspecific amplicons having the same size of the expected PCR products. A reliable but time consuming and quite labor- intensive verification method is a Southern blot assay, whereby the amplicon is separated by gel electrophoresis, transferred onto a nitrocellulose or nylon membranes and hybridized to a specific DNA probe. Nested PCR based on two successive PCR reactions. İn the second reaction, the PCR product is reamplified using second set of primers specifically designed for an inner region of the original target sequence. Therefore it allows discrimination between specific and non-specific amplification signals. The most reliable way to confirm the identity of the PCR products is its sequencing (Nollet, 2011).

In multiplex PCR, several primer pairs are included to permit the simultaneous detection of multiple target sequences. A multiplex PCR assays simultaneously amplifying the commonly used selectable marker genes, i.e., aadA, bar, hpt, nptII, pat encoding, respectively, for aminoglycoside-3′-adenyltransferase, *Streptococcus viridochromogenes* phosphinothricin-N-acetyltransferase, hygromycin phosphotransferase, neomycin phosphotransferase, *Streptococcus hygroscopicus* phosphinothricin-N-acetyltransferase, and a reporter gene uidA encoding β-d-glucuronidase, were developed as a reliable tool for qualitative screening of GM crops. This assay could be immensely used to test unintentional mixing of GM seeds with non-GM seed lots (Randhawa, 2009).

Recently, a multiplex polymerase chain reaction assay coupled to capillary gel electrophoresis for amplicon identification by size and color (multiplex PCR-CGE-SC) was developed for simultaneous detection of 6 cotton and 5 maize targets (two endogenous genes and 9 GM events) in two multiplex PCRs and a single CGE. The CGE assay accomplishes higher resolutions compared with agarose gel electrophoresis and has sensitivity and the reproducibility similar to QPCR. İn addition, the multiplex PCR-CGE-SC approach has high throughput and automation capabilities (Nadal, 2009).

Recently, a robust high-throughput analytical approach named multiplex microdroplet PCR implemented capillary gel electrophoresis (MPIC) was developed for high-throughput analysis of multiple DNA targets. This assay combines the advantages of bipartite primers, microdroplet PCR and CGE for multiple target DNA analysis, and at least 24 different targets can be simultaneously detected and identified (Guo, 2011).

The qualitative analysis procedure of a GMO is illustrated in Figure 3.2. DNAs were extracted from sample and analyzed by the PCR method. If the analysis of the endogenous gene in the food sample shows a negative result when compared to the control, GMO analysis of the sample is impossible. The endogenous reference gene must be species specific and not show allelic variation among various cultivars and have low or stable copy number in haploid genome. However, if PCR shows a positive result, further analysis methods, including screening PCR and event-specific PCR, should be performed to determine whether a sample contains or not contains GMOs.

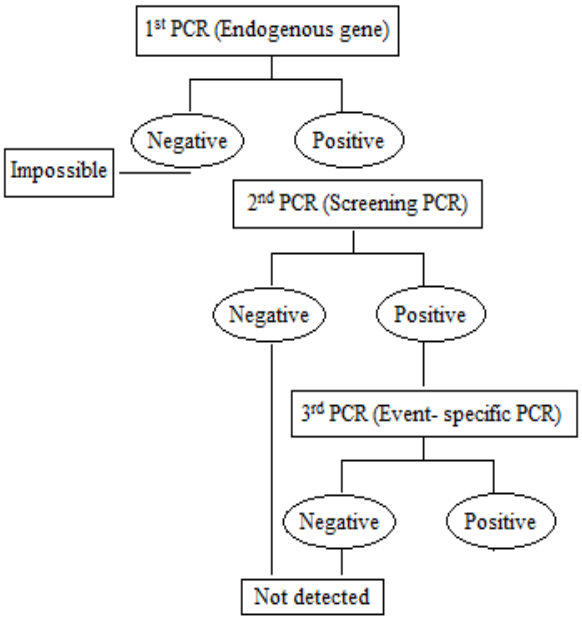


Figure 3.2 : Qualitative analysis procedure of a GMO.

3.1.1.1.1 Screening PCR

Screening PCR methods are based on detection of genetic elements common to many GMO events, which are not present in the conventional crop. Genetic control elements such as the cauliflower mosaic virus 35S promoter and/or the cauliflower mosaic virus 35S terminator or *Agrobacterium tumefaciens* nopaline synthase terminator are present in many GMOs currently on the market. The most commonly used cloning vectors are plasmids containing a gene coding for resistance to ampicillin (bla) antibiotics, or neomycin/ kanamycin (nptII) antibiotics. Consequently, Screening PCR targeting the p35S, t35S, tNOS, bla or nptII, have wide applications for screening for genetically modified material (Holst-Jensen, 2003). However, These sequences also occur naturally in plants and soil micro-organisms, therefore a positive result will not necessary confirm the presence of GMO, but will suggest that it is probable (Anklam et al., 2002). To definitively confirm the presence of a GMO, a sample with a positive signal in 35S and/or NOS screening should be further analysed using a construct-specific or event-specific method (Griffiths et al., 2003).

3.1.1.1.2 Gene specific PCR

Gene specific PCR methods target inserted gene coding for desirable traits. Typically structural genes are amplified in these methods such as Cry1A(b) coding for

endotoxin B1 from Bt, or the enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene coding for an enzyme conferring herbicide tolerance to the GM crops. Both gene specific and screening PCR methods are based on the detection of naturally present sequences in the environment for example promoter or terminator sequences from viruses or sequences coding for toxins from soil bacterium. Therefore, they could lead to increase of false positive results. Additionally these methods demonstrate only the presence of a genetically modified crop but they are not suitable for identification of the specific GMO. Because the gene or element may be present in more than one GMO, and their copy number may also vary from one GMO to another (Nollet, 2011).

3.1.1.1.3 Construct specific PCR

Construct specific PCR target junctions between two adjacent construct elements such as between the promoter and the gene of interest. An advantage of this method is that a positive results will only observed in the presence of GM crops (Adungna, 2008). However, more than one GMO can be shared same gene construct for example pV-ZMBK07 and pVZMGT10 into the following GM maize: Mon809 (1 copy of both), Mon810 (1 copy of the former), Mon832 (1 copy of the latter) (Holst-Jensen, 2003).

3.1.1.1.4 Event specific PCR

Event specific PCR target the integration locus at the junctions between recipient genome and the inserted DNA. When the same gene construct is used to produce different GM crops, this will be the only strategy to distinguish between GM crops containing the same gene construct (Tripathi, 2005). However, the method is not suitable to identify gene stacked events. Gene stacking technology allows the integration of multiple trait genes into a single plant line. When two different GMOs are cross fertilized, the new generation of plants exhibits the traits of the parent lines. Therefore, the new hybrid generation will be indistinguishable from its two parents with PCR method. GMOs with stacked genes are not regulated in the USA if both parent GMOs are authorized. It is treated as a new GMO and requires separate authorization in Europe. On the other hand, the gene stacked events are very rare when compared the other events according to the Database of International Service for the Acquisition of Agri-biotech Applications.

3.1.1.2 Quantitative PCR methods for GMO detection

In principle, quantification of GMOs is performed using quantitative PCR methods. In the quantitative PCR methods, the number of initial template molecules can be calculated based on the amount of the products through the standard curves.

The early quantitative PCR tests were based on quantitative competitive PCR (C-PCR), but QPCR is the most widely used method for GMO detection and represents the most powerful current means of quantifying GM crops (Buh Gasparic et al., 2008).

The quantitative competitive PCR method relies on the coamplification of unknown amounts of the template DNA originating from the sample and of known amounts of an internal control template in the same reaction tube by the same primer pair. In this method, control sequence is shorter (<40 bp.) -when compared to the target DNA sequence to be amplified and has the same sequence to which the primers may anneal. Sample is amplified with increasing amounts of competitor. Quantification is carried out by comparing the equivalence point at which the amplicon from the competitor gives the same signal intensity as the target DNA on stained agarose gels (Anklam, 2002). The quantitative competitive PCR method is less expensive than the realtime technology, but the necessary dilution series is considerably more time consuming.

QPCR allows for the real-time monitoring of the amplification reaction during each stage of the PCR. This is done via fluorometric measurement. In these methods the amount of amplicon synthesized during PCR is estimated directly by measurement of fluorescence in the PCR reaction.

Currently, several types of QPCR fluorogenic signal reagents are available for quantitative purposes for instance sequence unspecific DNA binding dyes (e.g., SYBR Green I), fluorescence resonance energy transfer (FRET) probes , TaqMan probes, LNA (locked nucleic acid) probe (Salvi et al., 2008), Plexor technology (BuhGasparic et al., 2008), light upon extension (LUX) probe (Nazarenko et al., 2002), molecular beacons (Andersen et al., 2006) and their derivatives (Amplifluor, Sunrise, and scorpion primers) (Whitcombe et al., 1999; Thelwell et al., 2000; Li et al., 2002). Among them, TaqMan probes and SYBR Green I are the most commonly used QPCR chemistries.

With the use of fluorescence it becomes possible to measure exactly the number of cycles that are needed to produce a certain amount of PCR product. This amount corresponds to the amount producing a fluorescence signal clearly distinguishable from the background signal and measured well before the plateau effect becomes a problem. The number is called the Ct value. Then by comparison of Ct values for the GM crop target sequence and the reference gene, it becomes possible to estimate the ratio of the GM target sequence to the reference sequence in terms of difference in number of cycles needed to produce the same quantity of product. Since one cycle corresponds to a doubling of the amount of product, a simple formula can be presented to estimate the ratio in percent. While realtime PCR requires more sophisticated and expensive equipment than competitive PCR, it is faster, automated and more specific. Presently, QPCR can be considered as the most powerful tool for the detection and quantification of GM crops and products.

If a product has been shown to contain GMO(s), the next step is to assess compliance with the threshold level 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 Q PCR.

Generally, the purpose of GMO quantification is to calculate the fraction of a certain species that comes from GM materials relying on quantitative PCR (Buh Gasparic et al., 2010). In the quantitative PCR assay, the number of initial template molecules can be calculated based on the amount of the products through the standard curves.

Quantification of GMOs can be either absolute or relative depending on the type of assay used. Absolute quantification is the real-time PCR analysis of choice for researchers who need to determine the actual copy number of the target under investigation. Absolute quantification is achieved by using a standard curve, constructed by amplifying known amounts of target DNA in a parallel set of reactions. Absolute quantification requires that the exact quantity of standards with a defined copy number or content of GM-derived DNA are used to construct a standard curve. For GMO quantification analysis, the choice of reference materials or calibrators used to generate the standard curves is important. Generally, genomic DNAs extracted from the certified reference materials (CRMs) from Institute of Reference Material and Measurement (IRMM) have been used. Certified GMO reference materials are needed for calibrating the methods used to quantify the GM

content and for controlling the quality of measurements. These certified samples consist of conventional seed flour fortified with genetically modified seed flour at a given w/w proportion. In addition to CRMs, so some researchers have produced their own calibration standards using purified genomic DNA or target DNA sequences cloned into plasmids (Tavernier et al., 2004).

Following amplification of the standard dilution series, the standard curve is generated by plotting the log of the initial template copy number against the Ct generated for each dilution. The plot of these points should generate a straight line. This line is the standard curve. Comparing the Ct values of the unknown samples to this standard curve allows the quantification of initial copy numbers.

Ideally, a standard curve will consist of at least 4 points, and each concentration should be run at least in duplicate (the more points the better). The range of concentrations in the standard curve must cover the entire range of concentrations that will be measured in the assay. In addition, the curve must be linear over the whole concentration range. The linearity is denoted by the R squared (Rsq) value (R 2 or Pearson Correlation Coefficient) and should be very close to 1 (> 0.985). A linear standard curve also implies that the efficiency of amplification is consistent at varying template concentrations.

Relative quantification is another widely used strategy. This method uses no known amount of standards but it compares the relative amount of the GMO target sequence to the reference gene sequence. Relative quantification is achieved by a combination of two absolute quantification reactions: one for the GMO-specific gene and a second for the endogenous reference gene (for use as a "normaliser"). 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 such in different lines of the same species and being as amplifiable as the GMO traits in analysis.

Standard curves are obtained for both the target and endogenous reference. For each experimental sample, the amount of target and endogenous reference is determined from the appropriate standard curve. The amount of target is normalised with the endogenous reference quantity to obtain the relative concentration of the target. A validation experiment must first be performed that demonstrates that the efficiency of target gene and the reference gene are approximately equal. A linear relationship is

established on the basis of the difference in Ct value of the reference gene and the GMO target, respectively, using e.g. certified reference materials covering a range of defined concentrations of the GMO material. The assumption inherent in this method is that the amplification efficiencies of the reference gene and the GM amplicon are the same in all subsequent experiments for all samples analysed. The approach therefore needs to be very well validated (Cankar et al., 2006). To meet statistical requirements, the standard curves should include at least 4 different concentration points. Each point of the standard curve, and the sample, should be loaded at least in triplicate.

3.2 Protein Based Methods

Immunoassay is the most common protein based method for detection and quantification of foreign proteins introduced through genetic transformation of plants. Immunoassay is based on the specific binding between an antigen and an antibody. The antibodies can be polyclonal or monoclonal. Western blot, Enzyme-Linked Immunosorbent Assay (ELISA) and lateral flow sticks have been used for the analysis of protein products of GM crops (Farid, 2002).

In ELISA antigens from the sample are attached on a solid phase. Antigen and antibody react and produce a stable complex, which can be visualized by addition of a second antibody linked to an enzyme. The subsequent reaction produces a detectable signal, most commonly a color change which can be measured photometrically or recognised by naked eye. The intensity of color indicates the amount of the protein present. It assumes more than one format: a micro well plate (or strip) format, and a coated tube format (Ahmed, 2002). Recently, a novel Immuno-PCR method that combines the specificity of an ELISA reaction with the sensitivity of PCR amplification was developed (Allen 2006). A sandwich enzyme-linked immunosorbent assay (S-ELISA) method for the phosphinothricin-N-acetyltransferase (PAT) encoded by the Bialaphos resistance (bar) gene in GM pepper was developed, showing a detection limit of 0.01 μg/mL in real samples examination (Shim, 2007).

Lateral flow strip or strip test is a version of ELISA using strips rather than micro titer wells. Protein strip tests are simple, fast, cheap and reliable, making them a

complementary tool to the PCR-based GMO detection methods (Van Duijn et al., 2002).

In western blotting, proteins are separated using polyacrylamide gel electrophoresis (PAGE). The proteins are then transferred to nitrocellulose membrane to which they bind. Nitrocellulose membrane is stained with a specific labeled antibody. The antibody may be labeled with 125I and the signal is detected with autoradiography. The detection limits of the western blots vary between 0.25% for seeds and 1% for toasted meal (Adunga, 2008).

Industrial processing easily denatures proteins. Therefore, protein-based methods are not appropriate for processed foods. In addition, developing specific antibodies have high costs and antibodies cannot be synthesized simply in comparison to oligonucleotides. They cannot discriminate between different transgenic events that express similar protein characteristics. Also GM products might be produced only during certain developmental stages or in certain plant parts and such GMOs are unlikely to be detected with protein based methods.

4. MATERIALS AND METHODS

4.1 Sampling and the Production of the Reference Material

Analyzed samples involve raw materials consumed as food and feedstuff and processed foods. Samples utilized in this study were given in Table 4.1. 17 35S, 2 FMV and 2 NOS positive and 75 GMO negative food samples (Table 5.8) that were already screened for presence of GMO by accredited food control laboratories (Environmental Industrial Analysis Laboratory Inc., Control Laboratory Inc, Quality System Laboratory Inc) were used for validation studies.

35S, FMV and NOS targeted QPCRs were applied on the positive samples that were supplied by the accredited laboratories. The PCR products were purified by using GF-1 Clean-Up Kit (Vivantis, Malaysia). Sequence analysis of the purified PCR products confirmed that amplified PCR products were the intended target gene regions. These purified PCR products were then used as reference DNAs.

Table 4.1 : Food sample types for validation studies.

Sample Number	Sample Type
1-4	Meatball
5-10	Soybean oil
11-36	Soybean flour
37-45	Corn
46-68	Corn oil
69-72	Tallow oil
73-79	Cat food
80-84	Dog food
85-94	Varieties of bread
95-96	Baklava

4.2 DNA Isolation

5 different silica column based DNA extraction protocols (Table 4.2) that were different in cell disruption strategy were tried on the soybean and maize samples.

The first DNA isolation method was the standard hexadecyltrimethylammonium bromide (CTAB) methodology (Yang et al., 1998). CTAB is a cationic detergent that disrupts protein and lipid molecules, and precipitates carbohydrate molecules. The second one was modification of first CTAB methodology that includes bead beating for physical cell disruption. The third methodology was based on NaOH- HCl treatment (Ozsensoy et al., 2008). In this method high base and high acid concentration were used to destroy the cells and tissues rapidly. The fourth one was modification of the third methodology that includes proteinase K and CTAB treatment. The fifth protocol, which was completely based on physical and chemical disruption, includes bead beating and CTAB treatment. In all of the methodologies, guanidium thiocyanate was used for PCR inhibitor inactivation and as a catiotrophic agent for DNA binding. The best results were obtained using the Protocol 5. Details of the Protocol 5 were given below. DNAs were extracted from the food samples other than the soybean and maize samples by using the Protocol 5.

1- 400 mg beat and 400 mg homogenized sample and 800 lysis solution (%2 CTAB (100 mM TrisHCl pH=8, 20 mM EDTA, 1.4 M NaCl) was added into 2 ml eppendorf tube, respectively.
2- In order to homogenization of sample, the mixture was centrifuged at 4500-6000 rpm for 1 minute.
3- The mixture was incubated at 95 °C for 10 minutes.
4- The mixture was centrifuged at 14000 rpm for 2 minutes and 400 µl supernatant was transferred into new 2 ml microfuge tubes.
5- 800 µl binding solution (6.75 M Guanidinium thiocyanate, 15 mM Tris-Cl pH 8.0) and 400 µl isopropanol were added and the sample was vortexed.
6- 800 µl mixtures was added into DNA colon and centrifuged at 14000 rpm for 1 minute and the precipitate was discarded. This step was repeated for the centrifugation of whole sample.
7- 500 µl inhibitor solution (60% (5 M thiocyanate, 20 mM Tris-HCl, pH 6.6), 40% EtOH) was added into DNA colon and was centrifuged at 14000 rpm for 1 minute and the precipitate was discarded.
8- 500 µl wash solution (20 mM NaCl, 2 mM Tris-HCl, pH 7.5; 80% v/v Ethanol) was added into DNA colon and was centrifuged at 14000 rpm for 1 minute and the precipitate was discarded.

9- 500 µl wash solution (20 mM NaCl, 2 mM Tris-HCl, pH 7.5; 80% v/v Ethanol) was added into DNA colon and was centrifuged at 14000 rpm for 1 minute and the precipitate was discarded.

10- The empty colon was centrifuged at 14000 rpm for 1 minute and transferred into new clean microfuge tube.

11- Finally, 100 µl elution solution (10 mM Tris-HCl pH 8) was added and incubated for 1 minute. The column was centrifuged at 14000 rpm for 1 min. The eluted DNA was stored at -20 °C.

Table 4.2 : DNA extraction protocols**.**

Protocol Number	Beat Beating	Proteinase K Treatment	NaOH - HCL Treatment	CTAB	Guanidinium Thiocyanate	References
1	-	+	-	+	+	D.Y. Yang et al., 1998
2	+	+	-	+	+	Modification
3	-	-	+	-	-	Y.Ozsensoy et al., 2008
4	-	+	+	+	+	Modification
5	+	-	-	+	+	Modification

4.3 The PCR Primers

Cauliflower Mosaic Virus 35S promoter, *Agrobacterium tumefaciens* Nopalin Synthase terminator and Figworth mosaic virus 35S promoter presents in more than 99% of the GMO events (Oliver, 2012). This is why these elements were chosen as PCR targets for GMO screening in this study. The previously described primers by ISO/FDIS (2005), Pan and others (2007) and Reiting and others (2007) were used for detection of 35S, NOS and FMV respectively. The original methodologies for FMV and 35S were based on conventional PCR amplification combined with product size determination via agarose gel electrophoresis. The original detection methodology for NOS terminator was based on real time PCR combined with the hydrolysis probes. The universal primer set that targets plant chloroplast DNA (the intergenic spacer region between the highly conserved tRNA val gene and the 16S rRNA gene) were derived from Al-Janabi and others (1994). All of the primers were synthesized by Oligo Macrogen, Korea. Primers used in this study are listed in Table 4.3.

Table 4.3 : Primer sets used in this study.

Region	Optimal Primer Sets	Sequence (5'-3')	T_m	Product size	References
35S promoter	Forward	GCTCCTACAAATGCCATCA	55.25	195	ISO/FDIS (2005)
	Reverse	GATAGTGGGATTGTGCGTCA	57.69		
FMV 35S promoter	Forward	AAGCCTCAACAAGGTCAG	54.39	196	Pan et al.,(2007)
	Reverse	CTGCTCGATGTTGACAAG	53.53		
NOS terminator	Forward	CATGTAATGCATGACGTTATTTATG	55.53	84	Reiting et al,. (2007)
	Reverse	TTGTTTTCTATCGCGTATTAAATGT	55.98		
Plant Chloroplast DNA	Forward	AGTTCGAGCCTGATTATCCC	58.72	297	Al-Janabi et al.,(1994)
	Reverse	GCATGCCGCCAGCGTTCATC	59.11		

4.4 Concentration Determination of Isolated DNA

The quality and amount of extracted DNAs was measured by using NanoDrop 2000 spectrophotometer (Thermo scientific, USA). Absorbance was measured at 260 nm and 280 nm. DNA absorbs UV light at 260 nm, but it is also required to know the absorbance values of proteins at 280 nm in order to evaluate the purity of DNA samples. The ratio of $A_{260/280}$ represents the purity of the samples. Pure DNA should have an $A_{260/280}$ ratio of approximately 1.8. If there is contamination with protein and aromatic substances, the $A_{260/280}$ value will be below 1.6 and the $A_{260/280}$ value above 2 indicates possible contamination with RNA (Pauli et al., 2000).

4.5 QPCR

The primer sets and their targets were given in Table 5.3. SsoFast™ EvaGreen® Supermix (dNTPs, Sso7d fusion polymerase, $MgCl_2$, EvaGreen dye) and Roche LightCycler® 480 System were utilized for all reactions. Reaction mixes contained 50 ng template DNA, 0.25 µM of each primer and 2.5 µM $MgCl_2$. The following thermocycling program was applied: 95 °C for 10 min, 45 cycles of 10 s at 95 °C and 20 s at 65 °C and 25 s at 72 °C. Melt-curve analysis was performed from 65 °C to 95 °C at 0.02 °C/sec ramp rate and the continuous fluorescence acquisition mode to determine T_m of the amplified products. QPCR runs were analyzed using Roche LightCycler® 480 Real Time PCR Software.

All of the Real time PCR reactions were repeated for three times. The FMV negative maize sample no 42 (Table 5.6) was used as a negative control in FMV targeted PCRs since FMV was only detected in the maize samples. The 35S and NOS

negative soybean flour sample no 26 (Table 5.6) was used as a negative control in 35S and NOS targeted PCRs. A chicken meat DNA was used as a negative control in plant targeted PCRs.

4.6 Sequence Analysis

The purified PCR products from the GMO positive were sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA). The obtained sequences were analyzed in Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html) and manually checked for reading errors. Homology searches of the sequences in DNA databases were performed with FASTA provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html). Gene sequences showing 97% or higher similarity to the genes that are already presents in the universal DNA data bank were considered as the same gene in the data bank.

5. RESULTS AND DISCUSSION

5.1 DNA Isolation for GMO Detection Analysis

High quality DNA is necessary to obtain sensitive and efficient results in PCR based methodologies (Wilson, 1997). In this study, we tried 5 different silica column based DNA extraction protocols on soybean and maize samples to obtain DNA with high quantity and quality. Protocol 1 was standard CTAB methodology for DNA isolation (Yang et al., 1998) that includes proteinase K treatment. CTAB is a cationic detergent that disrupts protein and lipid molecules, and precipitates carbohydrate molecules. Protocol 2 was modification of Protocol 1. Protocol 2 additionally includes bead beating for physical cell disruption. Protocol 3 was based on NaOH-HCl treatment (Ozsensoy et al., 2008). In this method high base and high acid concentration were used to destroy the cells and tissues rapidly. Protocol 4 was proteinase K and CTAB treatment added modification of Protocol 3. Protocol 5, which was completely based on physical and chemical disruption, includes bead beating and CTAB treatment. In all of the methodologies, guanidium thiocyanate was used for PCR inhibitor inactivation and as a catiotrophic agent for DNA binding.

The current methodologies of DNA extraction for GMO detection must result in at least 1.5 μg DNA with $A_{260/280}$ ratios between 1.6 and 2.0 (Elsanhoty et al., 2011). The results obtained in this study were given in Table 5.1. $A_{260/280}$ ratios of DNA extracts from all of the methods were in the desired range. The measured DNA concentrations were multiplied by the elution volume of 100 μl to calculate the amount of DNA obtained. All of the protocols were resulted in DNA amounts higher than 15 μg DNA, which is at least 10 times higher than the minimal limit (Elsanhoty et al., 2011). The best results in terms of DNA concentration were obtained from Protocols 2 and 5. The main difference between Protocols 2-5 and the other methodologies was the bead-beating step.

Spectrophotometer is a rapid and inexpensive method to measure DNA concentration, but it tends to overestimate the DNA concentration (Demeke et al., 2009). In this study, measurement of the concentration and quality of extracted DNA were examined by a spectrophotometer. On the other hand, another important aspect is the integrity of the DNA. The integrity assesment of the extracted DNAs were not determined using the systems such as Bioanalyzer (Agilent Technologies).

Table 5.1 : Concentrations and $A_{260/280}$ ratios of DNAs that extracted from soybean and maize samples using 5 different protocols.

	Soybean		**Maize**	
Protocol Number	**Conc. (ng/µl)**	**A260/280**	**Conc. (ng/µl)**	**A260/280**
Protocol 1	490.1± 7.5	1.84±0.11	382.1± 11.6	1.67±0.14
Protocol 2	600.5± 6.9	1.72±0.13	497.3± 15.4	1.93±0.03
Protocol 3	180.0± 4.7	1.79±0.13	195.4± 5.9	1.89±0.07
Protocol 4	204.5± 5.2	1.66±0.09	201.9± 6.7	1.76±0.11
Protocol 5	610.4± 9.6	1.81±0.04	500.3± 8.4	1.74±0.12

Since proteins absorb at 280 nm, the ratio $A_{260/280}$ is used to estimate the presence of the proteins in DNA extracts. On the other hand, the presence of other types of PCR inhibitors such as carbohydrates, phenols, aromatic compounds and heavy metals may also affect the PCR results. To comparatively evaluate effect of the DNA quality obtained by different protocols on the QPCR efficiency, the same amount of template DNAs (200 ng) were used in QPCR. The universal plant chloroplast DNA targeted PCR primers were used in QPCR trials. The obtained C_t values indicated the presence of PCR inhibitors because the DNA concentrations and purities were the same for all diluted templates obtained from different protocols. The amplification charts, melting curves and melting peaks obtained from 5 different protocols were shown in Figure 5.1. The obtained C_t values were also given in Table 5.2. All of the templates were resulted in plant chloroplast DNA specific T_m values. C_t values obtained using Protocols 2 and 5 were 2 cycles lower than the other protocols. This showed that these protocols were more successful in eliminating the PCR inhibitors. In this thesis, protocol 5 was selected for further studies. Advantage of the protocol was the eliminating enzymatic digestion steps which make protocol more time consuming and expensive.

Table 5.2 : T_m and Ct values of soybean and maize samples.

	Soybean	Maize	Soybean	Maize
	Ct	Ct	T_m	T_m
Protocol 1	16.1 ± 0.3	18.6 ± 0.1	82.5 ± 0.1	82.4 ± 0.2
Protocol 2	13.4 ± 0.6	16.1 ± 0.3	82.3 ± 0.3	82.2 ± 0.1
Protocol 3	17.7 ± 0.3	20.4 ± 0.5	82.1 ± 0.1	82.2 ± 0.3
Protocol 4	19.6 ± 0.5	22.7 ± 0.4	82.4 ± 0.2	82.5 ± 0.1
Protocol 5	14.0 ± 0.1	16.8 ± 0.2	82.3 ± 0.1	82.3 ± 0.2

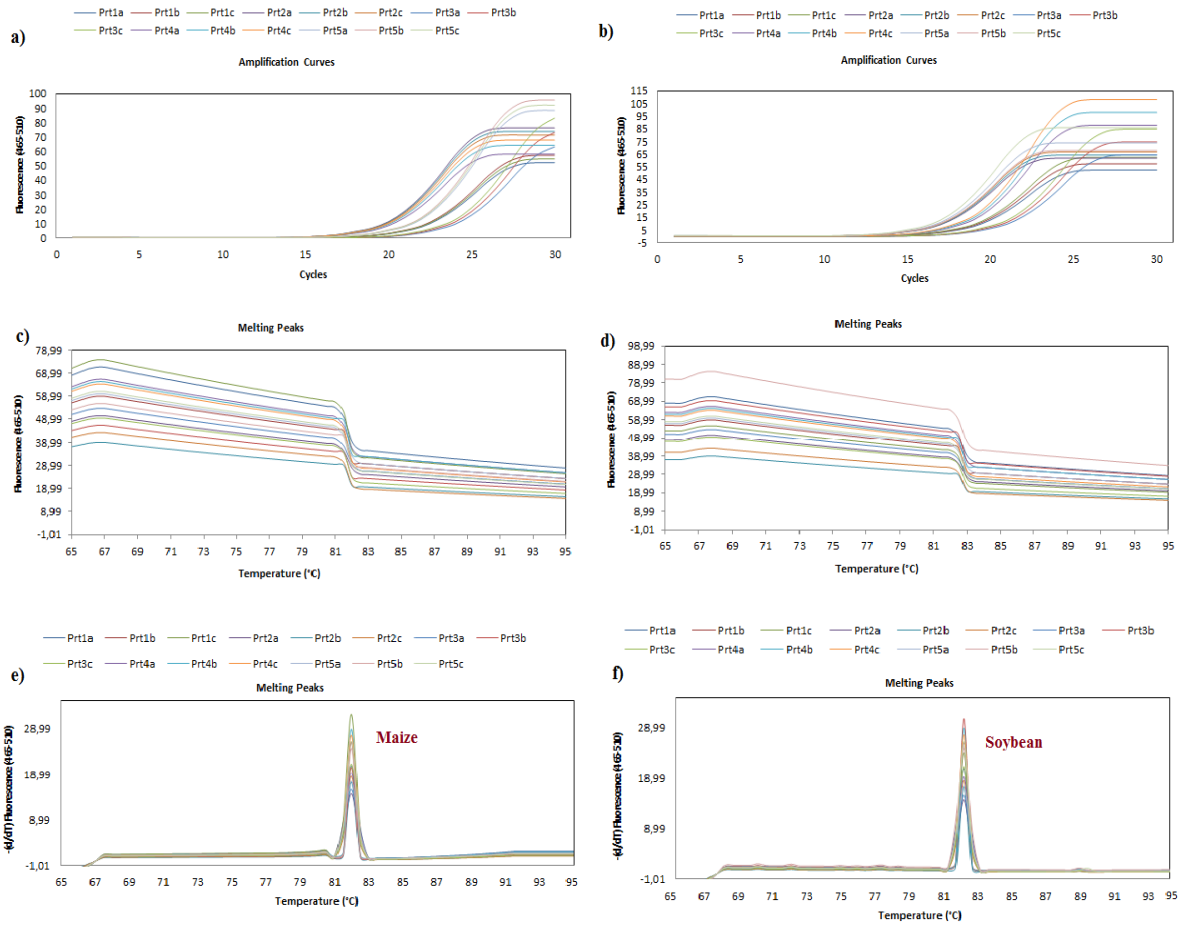


Figure 5.1 : The amplification charts (a,b), melting curves (c,d) and melting peaks (e,f) of DNAs that extracted from Maize and Soybean sample using 5 different protocols, respectively.

DNAs from the sample types other than maize and soybean samples were also isolated using Protocol 5. The results were given in Table 5.3. The results showed that the obtained DNAs were in the desired ranges in terms of DNA purity and

concentration. The DNA extraction efficiencies for the processed food samples were lower than the ones from the raw materials.

Table 5.3 : Concentration and $A_{260/280}$ ratio of DNAs from all samples using protocol 5.

Sample	Concentration	$A_{260/280}$	Sample	Concentration	$A_{260/280}$
1	345.8 ± 8.2	1.82 ± 0.01	**33**	872.4 ± 54.2	1.74 ± 0.02
2	374.4 ± 8	1.76 ± 0.04	**34**	734 ± 14.2	1.82 ± 0.03
3	397 ± 7.5	1.77 ± 0.04	**35**	847.4 ± 7.9	1.79 ± 0.01
4	368.1 ± 3.1	1.74 ± 0.05	**36**	876.5 ± 39.5	1.62 ± 0.07
5	454.1 ± 10.4	1.69 ± 0.05	**37**	732.5 ± 13	1.73 ± 0.05
6	419.3 ± 10.6	1.86 ± 0.01	**38**	922.8 ± 2.8	1.78 ± 0.03
7	366.1 ± 8.6	1.71 ± 0.02	**39**	862.5 ± 5.8	1.9 ± 0.07
8	500.4 ± 2.02	1.74 ± 0.03	**40**	1011.3 ± 18.4	1.63 ± 0.04
9	594.3 ± 15.3	1.76 ± 0.02	**41**	955.5 ± 8.7	1.69 ± 0.04
10	458.7 ± 8.7	1.86 ± 0.01	**42**	933.6 ± 48.7	1.98 ± 004
11	756.1 ± 17.9	1.68 ± 0.03	**43**	864.9 ± 11.5	1.71 ± 0.07
12	773.7 ± 12.4	1.6 ± 0.03	**44**	941.6 ± 16.5	1.71 ± 0.04
13	849.4 ± 1.6	1.6 ± 0.02	**45**	1028.6 ± 25.7	1.69 ± 0.02
14	911.4 ± 7.9	1.83 ± 0.01	**46**	580,5 ± 4	1.9 ± 0.06
15	882.4 ± 16.4	1.79 ± 0.03	**47**	656.5 ± 9.6	1.8 ± 0.02
16	730 ± 16.1	1.6 ± 0.04	**48**	308.1 ± 5.5	1.6 ± 0.04
17	779.1 ± 21.7	1.79 ± 0.05	**49**	441.6 ± 1.4	1.68 ± 0.05
18	796.9 ± 15.5	1.98 ± 0.01	**50**	629 ± 12.6	1.93 ± 0.01
19	846.3 ± 17.8	1.66 ± 0.07	**51**	553.8 ± 12.4	1.82 ± 0.03
20	896.6 ± 9.8	1.99 ± 0.01	**52**	337.1 ± 10.3	1.78 ± 0.07
21	724.3 ± 16.6	1.7 ± 0.03	**53**	712.9 ± 21.2	1.62 ± 0.04
22	825.5 ± 13.6	1.9 ± 0.01	**54**	332.2 ± 4.8	1.83 ± 0.05
23	718,4 ± 189	1.67 ± 0.06	**55**	556.8 ± 10	1.65 ± 0.02
24	827.7 ± 13.7	1.59 ± 0.07	**56**	558,8 ± 12	1.95 ± 0.05
25	893.4 ± 9.4	1.72 ± 0.03	**57**	292.7 ± 4.9	1.83 ± 0.07
26	717.8 ± 6.2	1.92 ± 0.01	**58**	245.4 ± 8	1.65 ± 0.01
27	828 ± 4.8	1.88 ± 0.02	**59**	619.2 ± 8.9	1.62 ± 0.07
28	861,2 ± 48.3	1.73 ± 0.01	**60**	726.1 ± 12.1	1.68 ± 0.03
29	763.3 ± 45.8	1.70 ± 0.07	**61**	450 ± 3.3	1.8 ± 0.03
30	717.4 ± 13.5	1.92 ± 0.04	**62**	677.7 ± 7.7	1.76 ± 0.05
31	739.8 ± 15.7	1.6 ± 0.09	**63**	464 ± 12.3	1.92 ± 0.02
32	930.7 ± 1.2	1.79 ± 0.02	**64**	740.7 ± 7.6	1.66 ± 0.03

Table 5.3 (continued) : Concentration and $A_{260/280}$ ratio of DNAs from all samples using protocol 5.

Sample	Concentration	$A_{260/280}$	Sample	Concentration	$A_{260/280}$
65	198.1 ± 10.6	1.91 ± 0.01	**81**	288 ± 9.2	1.83 ± 0.02
66	660 ± 15.7	1.97 ± 0.01	**82**	281.8 ± 9	1.65 ± 0.02
67	718.4 ± 8.7	1.69 ± 0.04	**83**	526.3 ± 9	1.94 ± 0.02
68	750,3 ± 5.7	1.71 ± 0.05	**84**	568.9 ± 4.7	1.84 ± 0.01
69	446.4 ± 6.5	1.91 ± 0.02	**85**	357.4 ± 5.3	1.69 ± 0.01
70	236.2 ± 6.9	1.75 ± 0.03	**86**	242.5 ± 6.8	1.61 ± 0.02
71	351 ± 4.7	1.64 ± 0.04	**87**	281 ± 7.8	1.80 ± 0.03
72	287.8 ± 9.4	1.89 ± 0.02	**88**	349.5 ± 3.7	1.86 ± 0.04
73	520 ± 4.3	1.80 ± 0.03	**89**	355.2 ± 9.2	1.67 ± 0.02
74	432.8 ± 5.1	1.73 ± 0.02	**90**	312 ± 8.8	1.90 ± 0.03
75	556.6 ± 8	1.69 ± 0.03	**91**	230.1 ± 6.2	1.87 ± 0.01
76	342.2 ± 18.8	1.71 ± 0.02	**92**	381.6 ± 6	1.61 ± 0.02
77	362.9 ± 10.8	1.89 ± 0.01	**93**	423.4 ± 2	1.86 ± 0.01
78	363.6 ± 9.7	1.63 ± 0.02	**94**	360.9 ± 3.7	1.63 ± 0.03
79	397.8 ± 7.5	1.63 ± 0.01	**95**	244.7 ± 10	1.92 ± 0.01
80	268.3 ± 7.8	1.93 ± 0.01	**96**	460.5 ± 7.4	1.86 ± 0.02

5.2 Reference Material Construction

FMV, NOS, 35S positive reference food samples were supplied by the accredited food control laboratories. The types of positive samples were given in Table 5.8. NOS positive sample no 23, FMV positive sample no 40 and 35S positive sample no 11 were used as a reference sample. Extracted DNAs from FMV, NOS, 35S positive food samples were amplified by using the target specific primer pairs (Table 4.3). DNA amplification curves were analyzed via the second derivative maximum method and Ct value was calculated based on the start of exponential DNA amplification. There was an inverse relationship between identified Ct value and the amount of target DNA present in the analyzed sample.

Each dsDNA has sequence-specific T_m degree. A negative first derivation curve of the fluorescence intensity (F) curve over temperature (T) produced by the instrument's software indicates the T_m of the PCR product (peak of the –dF/dT curve) and should be quite close to the predicted T_m of the PCR product (Dorak,

2007). In this study, melting curve analysis was performed after the amplification cycles and T_m of the targeted PCR products were calculated. Each reaction was performed for three times. The obtained amplification charts, melting curves and melting peaks were shown in Figure 5.2. Specific T_m degrees of the each amplicon were given in Table 5.4. The target specific melting peaks were obtained at 73 ± 0.38˚C for NOS, 80˚C ± 0.28˚C for FMV, 82.26 ± 0.29˚C for 35S and 82 ± 0.33˚C for plant specific reactions. In the reactions, the standard deviations were lower than 0.4 ˚C. It is generally accepted that the T_m obtained with Evagreen QPCR could vary between 0.5 and 1 ˚C for the same amplicon (Donohoe et al., 2000, Hermann et al., 2007). Therefore, to cover slight deviations in the T_m value between reference materials and samples due to analyte impurities, a standard deviation of ±1 ˚C on the normal T_m value will be applied, as the acceptance range, in further analysis. In addition, all of the Evagreen QPCR reactions generated a single specific signal without major additional amplification products.

The standard reference samples were prepared via purification of the PCR amplified target DNAs. Concentrations of the purified DNAs were determined using a spectrophotometer. The molecular weight of a single target DNA was calculated based on its DNA sequences. The gene copy numbers were calculated via dividing DNA concentrations by the molecular weights. Serial dilutions were done to obtain standard reference samples containing 10^0-10^{10} copies of the targeted gene.

Table 5.4 : T_m of the PCR amplified NOS, FMV, 35S and Plant DNA.

Target	**T_m (°C)**
NOS	73 ± 0.38
FMV	80 ± 0.28
35S	82.3 ± 0.29
Plant	82 ± 0.33

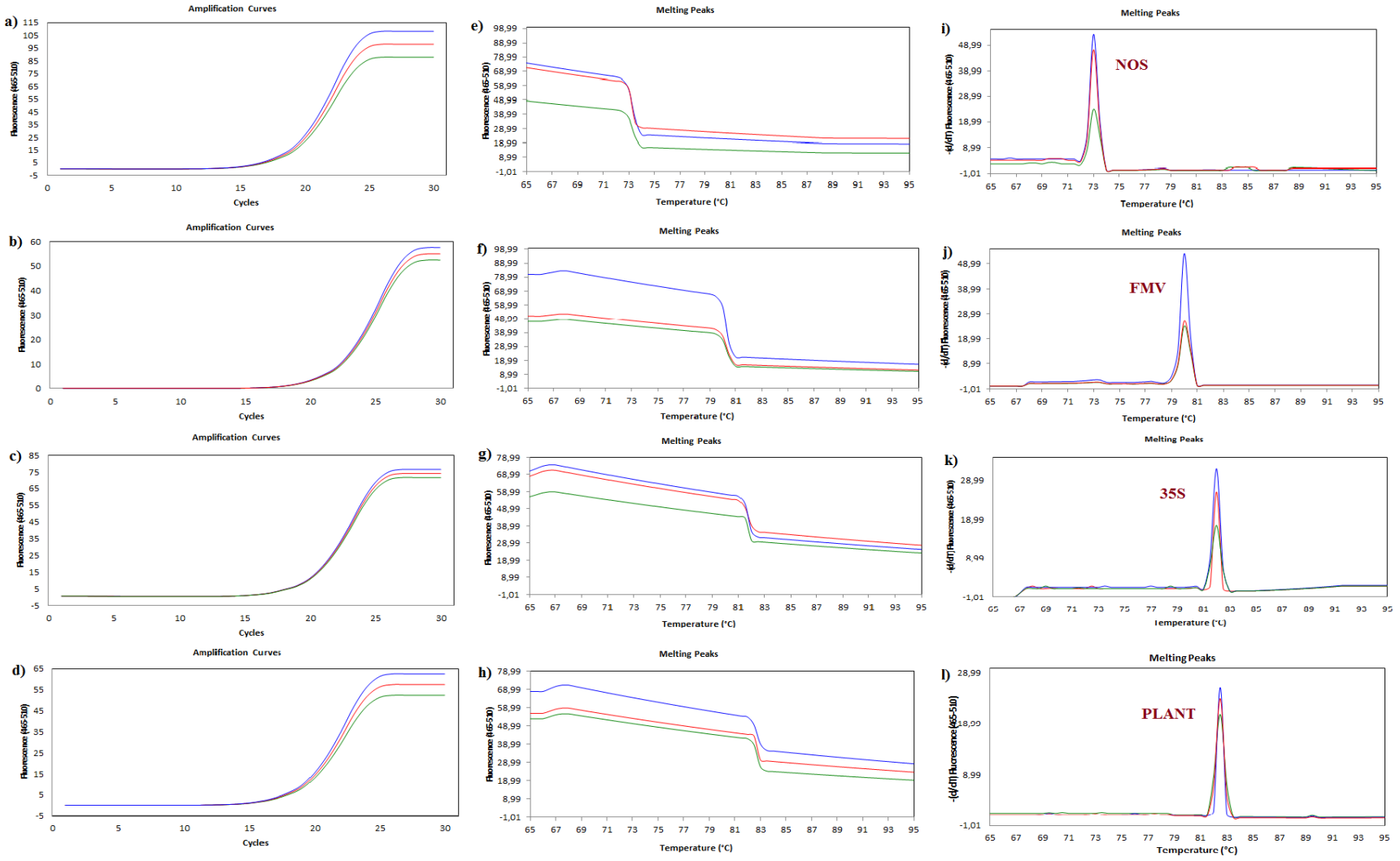


Figure 5.2 : The amplification charts (a, b, c, d), the melting curves (e, f, g, h) and the melting peaks (i, j, k, l) of NOS, FMV, 35S and Plant positive DNAs, respectively. First, second and third runs were shown in blue, red and green, respectively.

5.3 Multiple Detection of 35S, NOS and FMV

The same amounts (1000 copies) of different DNA templates were added to the initial duplex QPCRs trials. The favored DNA templates, which resulted in more abundant PCR products in duplex reactions, were determined via melting curve analysis. As seen in Figure 5.3, the FMV templates resulted in more PCR products. The 35S templates were favored in PCRs that contained the 35S and NOS templates.

The subsequent trials were carried out till only one type of T_m peak was obtained to determine the effect of different initial template amounts on the duplex QPCRs. 1000 copies of the dominant template and 100 copies of the less amplified template, 1000

copies of the dominant template and 10 copies of the less amplified template were used in the second and third trials respectively. The fourth trial, which gave only the T_m peak of the dominant template, contained 1000 copies of the dominant template and 1 copy of the less amplified template. The overall results showed that; two different T_m peaks were not obtained under 1/100 relative template concentrations but two different T_m peaks were obtained for each target above 1/100 relative template concentrations.

The obtained amplification curves, melting curves and melting peak charts of 1/1 relative template concentration in the duplex QPCR trails were shown in Figure 5.3. The amplification charts, melting curve and melt peak charts of 1/100, 1/10, 1/1 relative template concentration were shown in Figure A.2. The determined Ct and T_m values were given in Table 5.5.

In the binary QPCR trials, T_m of the melt curve profile can identify which target sequences were amplified by PCR. In the binary reactions, expected T_m values corresponding to 35S, FMV and NOS targets were at 82, 80 and 73 ˚C, respectively. As seen in Figure 5.3 and Figure A.1, all of the targets resulted in T_m values at the expected temperatures for all relative template ratios.

Two different melting peaks were clearly separated from each other in the NOS and 35S specific multiplex QPCR reactions and the average T_ms of the different peaks were significantly different. The melting peaks corresponding to NOS target were observed at 73.22 ± 0.13 ˚C (1/1 template ratio), 73.09 ± 0.21 ˚C (10/1 template ratio), 73.1 ± 0.22 ˚C (100/1 template ratio) and the melting peaks corresponding to

35S target were observed at 82.1± 0.08 ˚C (1/1 template ratio), 82.15 ± 0.13 ˚C (10/1 template ratio), 82.21 ± 0.12 ˚C (100/1 template ratio) (Figure 5.3 and Figure A.1).

Two melting peaks corresponding to NOS and FMV target were clearly seperated from each other for all relative template concentrations of NOS and FMV specific binary trials. The melting peaks corresponding to NOS target were observed at 73.13± 0.40 ˚C (1/1 template ratio), 73.09 ± 0.33 ˚C (10/1 template ratio), 73.03 ± 0.24 ˚C (100/1 template ratio) and the melting peaks corresponding to FMV target were observed at 80.5± 0.43 ˚C (1/1 template ratio), 80.21 ± 0.23 ˚C (10/1 template ratio), 80.05 ± 0.13 ˚C (100/1 template ratio) (Figure 5.3 and Figure A.1).

In the each relative template concentration of binary trials of FMV and 35S targets, two different melting peaks were observed at the expected temperatures. The melting peaks corresponding to FMV target were observed at 80.3± 0.17 ˚C (1/1 template ratio), 80.25 ± 0.2 ˚C (10/1 template ratio), 80.19 ± 0.23 ˚C (100/1 template ratio) and the melting peaks corresponding to 35S target were observed at 82.5± 0.15 ˚C (1/1 template ratio), 82.09 ± 0.11 ˚C (10/1 template ratio), 82.38 ± 0.25 ˚C (100/1 template ratio) (Figure 5.3 and Figure A.1). Therefore, all binary QPCR trials generate unique peaks in melting analysis and the T_m values of the PCR products differ less than 1 ˚C from the normal T_m value of the reference DNAs.

Table 5.5: T_m degrees and standard deviation of each target in the binary reactions.

Mixtures	Ct	T_m1 (°C)	T_m2 (°C)
1000 copies FMV + 1000 copies NOS (1/1)	16.3	80.5 ± 0.43	73.13 ± 0.40
1000 copies FMV +100 copies NOS (10/1)	18.01	80.21 ± 0.32	73.09 ± 0.33
1000 copies FMV + 10 copies NOS (100/1)	16.48	80.05 ± 013	73.03 ± 0.24
1000 copies 35S+ 1000 copies NOS (1/1)	17.5	82.10 ± 0.08	73.22 ± 0.3
1000 copies 35S +100 copies NOS (10/1)	21.63	82.15 ± 0.13	73.09 ± 0.21
1000 copies 35S + 10 copies NOS (100/1)	22.15	82.21 ± 0.12	73.1 ± 0.22
1000 copies FMV+ 1000 copies 35S (1/1)	22.1	80.3 ± 0.17	82.13 ± 0.15
1000 copies FMV +100 copies 35S (10/1)	23.05	80.25 ± 0.2	82.09 ± 0.11
1000 copies FMV + 10 copies 35S (100/1)	23.29	80.19 ± 0.23	82.38 ± 0.25

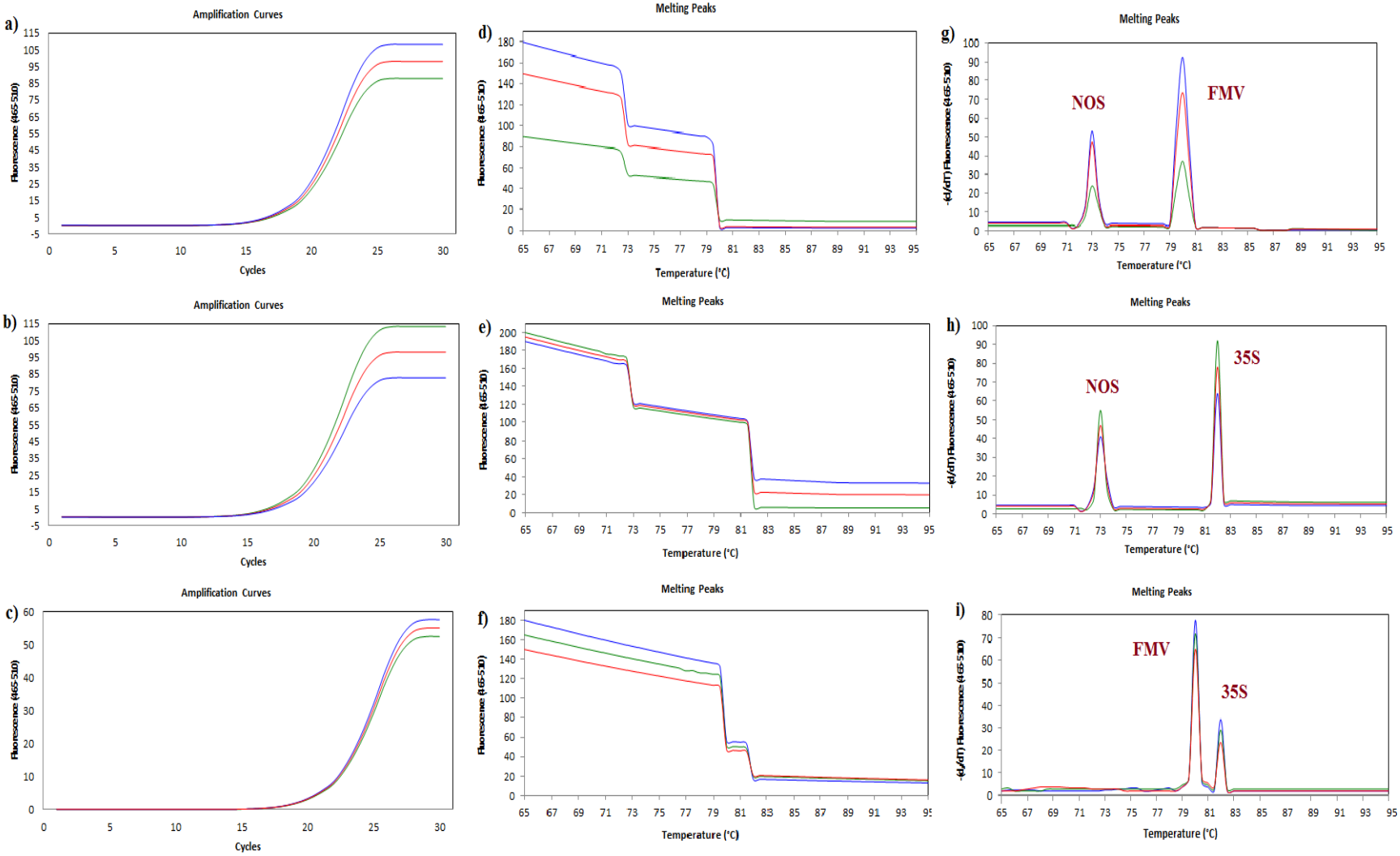


Figure 5.3 : The amplification charts (a,b,c), the melting curve charts (d,e,f) and the melting peaks (g,h,i) of 1/1 relative ratios of the binary DNA mixtures. First, second and third runs were shown in blue, red and green, respectively.

After the successful binary mixture trials, triple mixtures were prepared using 1000 copies of the each reference sample. The triplex QPCR trials were carried out to show that 3 primer pairs can work together in the multiplex QPCR and do not form non-specific PCR products or primer dimers. The triple combinations were applied to 1/1/1 relative copy number ratios of the reference samples. The obtained melting temperatures in triplex QPCRs were given in Table 5.6. The amplification curves, melting curves and melt peak charts of the triplex QPCRs were shown in Figure 5.4.

As seen in Figure 5.4, the NOS, FMV and 35S specific multiplex QPCR resulted in 3 different melting peaks at the expected temperatures. The melting peak corresponding to NOS, FMV and 35S targets were observed at 73.04± 0.13 ˚C, 80.21± 0.10 ˚C and 82.15± 0.08 ˚C, respectively. No additional amplification were observed in the multiplex reactions.

Since plant DNAs will always be the dominant target in GMO screening reactions, plants DNAs were not included in the binary and triple DNA mixtures to increase the detection sensitivities of the FMV, 35S and NOS targets. Plant specific QPCRs were carried out in GMO screening reactions as a positive PCR amplification control.

Table 5.6 : T_m degrees and standard deviation of each target in the triple reaction.

Target	T_m (°C)
NOS	73.04± 0.15
FMV	80.21± 0.12
35S	82.15± 0.08

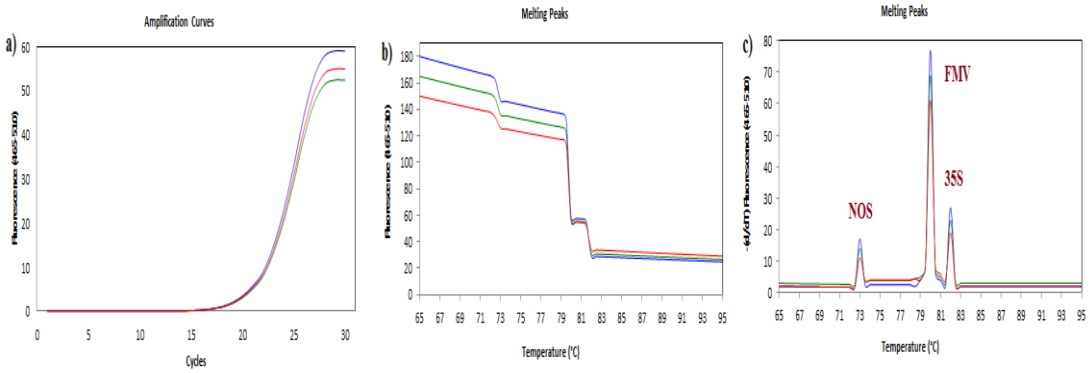


Figure 5.4 : The amplification chart (a), melting curve chart (b) and melting peak (c) of the triple DNA mixture. First, second and third runs were shown in blue, red and green, respectively.

5.4 Specificity and Sensitivity of the Detection Method

QPCR quantification standards were prepared using the purified PCR products from the reference samples. Molecular weights of the PCR products were calculated based on their DNA sequences. The gene copy numbers were calculated via dividing DNA concentrations by the molecular weights. Serial dilutions were done to obtain standard samples containing 10^0-10^{10} copies of the targeted gene.

To obtain the limit of detection (LOD), soybean samples that contain 1-100 copies of 35S and NOS per gr of the sample, and maize samples that contain 1-100 copies of FMV per gr of the sample were prepared. The limits of detections were 1 gene copies/gr food sample for the 35S, NOS and FMV targeted methodologies. On the other hand, since the standard mixtures were not obtained from an acredited reference laboratory, the detected LODs were rough estimations of the real LODs.

A DNA mixture of the 35S, NOS, FMV genes were prepared to test the specificity of the primers. The DNA mixture was amplified via QPCR with each specific primer pair. The specificity of the QPCR reactions were examined via sequencing of the each amplified PCR products. Homology searches of the obtained sequences were done using blast-n tool of National Center for Biotechnology Information. The blast analyses results and the sequence chromatograms were shown in Figure 5.5, 5.6, 5.7, 5.8 and Figure A.2.

Score	Expect	Identities	Gaps	Strand
285 bits(154)	7e-74	157/158(99%)	1/158(0%)	Plus/Plus

```
Query  1     CATCGTTGAAGATGCCTCTGCCGACAGTGGT-CCAAAGATGGACCCCCACCCACGAGGAG  59
             ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  5278  CATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAG  5337

Query  60    CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5338  CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT  5397

Query  120   CTCCACTGACGTAAGGGATGACGCACAATCCCACTATC  157
             |||||||||||||||||||||||||||||||||||||
Sbjct  5398  CTCCACTGACGTAAGGGATGACGCACAATCCCACTATC  5435
```

Figure 5.5 : Blast hit analysis of 35S promoter sequencing result and targeted Moss transformation vector pTFH22.4 DNA, complete sequence (|, indicates the homologous base pairs).

The homology search results were summarized in Table 5.7. Multiple sequence alignment was also carried out using ClustalW2 tool of the European Bioinformatics Institute (Figures 5.9 - 5.12). The results showed that the amplified sequences have at least 99% similarity to the intended targets. It is planning to confirm specificity of all obtained PCR products by sequencing in the more detailed study that will be done in the future.

Score	Expect	Identities	Gaps	Strand
250 bits(135)	2e-63	139/141(99%)	0/141(0%)	Plus/Minus

```
Query  1     CCAAAAGCCACAGGAGACCAATGAAGAATCTTCAATCAAAGTAAACTACTGTTCCAGCAC  60
             |||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  8813  CCAAAAGCTACAGGAGATCAATGAAGAATCTTCAATCAAAGTAAACTACTGTTCCAGCAC  8754

Query  61    ATGCATCATGGTCAGTAAGTTTCAGAAAAAGACATCCACCGAAGACTTAAAGTTAGTGGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8753  ATGCATCATGGTCAGTAAGTTTCAGAAAAAGACATCCACCGAAGACTTAAAGTTAGTGGG  8694

Query  121   CATCTTTGAAAGTAATCTTGT  141
             |||||||||||||||||||||
Sbjct  8693  CATCTTTGAAAGTAATCTTGT  8673
```

Figure 5.6 : Blast hit analysis of FMV sequencing result and targeted Expressionvector pMON99036, complete sequence (|, indicates the homologous base pairs).

Score	Expect	Identities	Gaps	Strand
294 bits(159)	1e-76	159/159(100%)	0/159(0%)	Plus/Plus

```
Query  1     TTGAATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGATGGCTATAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97260 TTGAATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGATGGCTATAT  97319

Query  61    TTCTGGGAGCGAACTCCAGGCGAATATGAAGCGCATGGGTACAAGTTATGCCTTGGAATG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97320 TTCTGGGAGCGAACTCCAGGCGAATATGAAGCGCATGGGTACAAGTTATGCCTTGGAATG  97379

Query  121   AAAGACAATTCCGAATCCGCTTTGTCTACGAACAAGGAA  159
             |||||||||||||||||||||||||||||||||||||||
Sbjct  97380 AAAGACAATTCCGAATCCGCTTTGTCTACGAACAAGGAA  97418
```

Figure 5.7 : Blast hit analysis of Plant sequencing result and targeted Beta vulgaris chloroplast sequence (|, indicates the homologous base pairs).

Score	Expect	Identities	Gaps	Strand
79.8 bits(40)	9e-13	40/40(100%)	0/40(0%)	Plus/Plus

```
Query  1     GAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAA  40
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  6417  GAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAA  6456
```

Figure 5.8 : Blast hit analysis of NOS sequencing result and targeted Moss transformation vector pTFH22.4 DNA, complete sequence (|, indicates the homologous base pairs).

Table 5.7 : The homology search results.

Target	Blast Hit	Accession Number	Similarity
35S promoter	Moss transformation vector pTFH22.4 DNA, complete sequence	dbj|AB758445.1|	99%
NOS terminator	Moss transformation vector pTFH22.4 DNA, complete sequence	dbj|AB758445.1|	100%
FMV promoter	Expression vector pMON99036, complete sequence	gb|JN400388.1|	99%
Plant	Beta vulgaris chloroplast sequence	gb|EF534108.1|	100%

```
Temp            ----------------------------------------------------------CCAAA 5
FMV             AATTCTCAGTCCAAAGCCTCAACAAGGTCAGGGTACAGAGTCTCCAAACCATTAGCCAAA 60
                                                                          *****

Temp            AGCCACAGGAGACCAATGAAGAATCTTCAATCAAAGTAAACTACTGTTCCAGCACATGCA 65
FMV             AGCTACAGGAGATCAATGAAGAATCTTCAATCAAAGTAAACTACTGTTCCAGCACATGCA 120
                *** ******** ************************************************

Temp            TCATGGTCAGTAAGTTTCAGAAAAAGACATCCACCGAAGACTTAAAGTTAGTGGGCATCT 125
FMV             TCATGGTCAGTAAGTTTCAGAAAAAGACATCCACCGAAGACTTAAAGTTAGTGGGCATCT 180
                ************************************************************

Temp            TTGAAAGTAATCTTGT-------------------------------------------- 141
FMV             TTGAAAGTAATCTTGTCAACATCGAGCAGCTGGCTTGTGGGGACCAGACAAAAAAGGAAT 240
                ****************

Temp            ------------------------------------------------------------
FMV             GGTGCAGAATTGTTAGGCGCACCTACCAAAAGCATCTTTGCCTTTATTGCAAAGATAAAG 300

Temp            ------------------------------------------------------------
FMV             CAGATTCCTCTAGTACAAGTGGGGAACAAAATAACGTGGAAAAGAGCTGTCCTGACAGCC 360

Temp            ------------------------------------------------------------
FMV             CACTCACTAATGCGTATGACGAACGCAGTGACGACCACAAAAGAATTAGCTTGAGCTCAG 420
```

```
Temp            ------------------------------------------------------------
FMV             GATTTAGCAGCATTCCAGATTGGGTTCAATCAACAAGGTACGAGCCATATCACTTTATTC 480


Temp            ------------------------------------------------------------
FMV             AAATTGGTATCGCCAAAACCAAGAAGGAACTCCCATCCTCAAAGGTTTGTAAGGAAG 537
```

Figure 5.9 : Clustal W analysis of obtained FMV promoter sequencing results and targeted FMV promoter sequence (*, indicates the homologous base pairs).

```
Tmp             TCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTT 60
Nos             ------------------------------------------------------------


Tmp             GCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATT 120
Nos             ------------------------------------------------------------


Tmp             AACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTA 180
Nos             ----------------------------------------------GAGTCCCGCAATTA 14
                                                               **************


Tmp             TACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGC 240
Nos             TACATTTAATACGCGATAGAAAACAA---------------------------------- 42
                **************************


Tmp             GCGGTGTCATCTATGTTACTAGATCGGG 268
Nos             ----------------------------
```

Figure 5.10 : Clustal W analysis of obtained NOS terminator sequencing results and targeted NOS terminator sequence (*, indicates the homologous base pairs).

```
Temp            ------------------------------------------------------------
Plant           AGTTCGAGCCTGATTATCCCTAAACCCAATGTGAGTTTTTCTATTTTTACTTGCTTCCCC 60


Temp            -------TTGAATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGATG 53
Plant           GCCGTGATCGAATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGATG 120
                       * **************************************************


Temp            GCTATATTTCTGGGAGCGAACTCCAGGCGAATATGAAGCGCATGGGTACAAGTTATGCCT 113
Plant           GCTATATTTCTGGGAGCGAACTCCAGGCGAATATGAAGCGCATGGATACAAGTTATGCCT 180
                **********************************************.***************


Temp            TGGAATGAAAGACAATTCCGAATCCGCTTTGTCTACGAACAAGGAA-------------- 159
Plant           TGGAATGAAAGACAATTCCGAATCCGCTTTGTCTACGAACAAGGAAGCTATAAGTAATGC 240
                *******************************************


Temp            ------------------------------------------------------------
Plant           AACTATGAATCTCATGGAGAGTTCGATCCTGGCTCAGGATGAACGCTGGCGGCATGC 297
```

Figure 5.11 : Clustal W analysis of obtained Plant sequencing results and targeted Plant sequence (*, indicates the homologous base pairs and arrows indicates primers).

```
Temp            ------------------------------------------------------------
35s             AGATTAGCCTTTTCAATTTCAGAAAGAATGCTAACCCACAGATGGTTAGAGAGGCTTACG 60


Temp            ------------------------------------------------------------
35s             CAGCAGGTCTCATCAAGACGATCTACCCGAGCAATAATCTCCAGGAAATCAAATACCTTC 120


Temp            ------------------------------------------------------------
35s             CCAAGAAGGTTAAAGATGCAGTCAAAAGATTCAGGACTAACTGCATCAAGAACACAGAGA 180


Temp            ------------------------------------------------------------
35s             AAGATATATTTCTCAAGATCAGAAGTACTATTCCAGTATGGACGATTCAAGGCTTGCTTC 240


Temp            ------------------------------------------------------------
35s             ACAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAAAGGTAGTTCCCACTGAATCAA 300
```

Figure 5.12 : Clustal W analysis of obtained 35S promoter sequencing results and targeted 35S promoter sequence (*, indicates the homologous base pairs).

5.5 Screening the Food Samples

The raw and processed food samples (Table 5.8), which were already analyzed by the accredited food control laboratories, were re-analyzed using the developed methodology. Total of 96 samples that include meatballs, soybean oil, soybean meal, corn, corn oil, tallow oil, cat and dog foods, chocolate, baklava and bread varieties were analyzed. The obtained results for each sample were given in Table 5.8. The amplification curves, melting curves and melt peak charts of some of the analyzed commercial samples that gave positive signal in NOS, FMV and 35S multiplex PCR reaction were shown in Figure 5.13. 17 35S, 2 NOS and 2 FMV positive samples were detected among the 96 screened samples. Our results were in 100% accordance with the results obtained by the accredited food control laboratories.

In the 6 different soybean oil samples, one 35S positive signal was obtained from the sample number 10. In this NOS, FMV and 35S multiplex reaction, a melting peak

was observed at 82.2 ˚C. In the 26 different soybean flour samples, 10 35S positive signal and 1 NOS positive signal was obtained. Among them, the sample number 19 was given in the Figure 5.13. As seen in the figure, a melting peak was observed at 82.91 ˚C. Among the 9 different maize samples, 2 35S positive signals and 2 35S and FMV positive signal were obtained. 35S and FMV positive sample number 44 and 35S positive sample number 45 were given in the Figre 5.13. In the 23 different maize oil samples, 1 NOS positive signal was obtained from the sample number 54. As seen in the figure, melting peak was observed at 73.12 ˚C in this reaction.

As result of the experiment, just the presence or absence of the targets in a sample can be discriminated in the study. Such a method would allow discrimination of samples that is possible to contain GMOs from those that are free of GMOs. However, positive samples can then be analyzed further to determine the strain of GMO present and the amount of targets to determine threshold percent for labeling.

Table 5.8 : The results of the screening samples.

Sample No	Sample Type	Plant Specific PCR	35S Specific PCR	NOS Specific PCR	FMV Specific PCR	35S, FMV, NOS Specific Multiplex PCR		
						35S	NOS	FMV
1		+	-	-	-	-	-	-
2		+	-	-	-	-	-	-
3		+	+	-	-	+	-	-
4	**Meatball**	+	-	-	-	-	-	-
5		+	-	-	-	-	-	-
6		+	-	-	-	-	-	-
7		+	-	-	-	-	-	-
8		+	-	-	-	-	-	-
9		+	-	-	-	-	-	-
10	**Soybean oil**	+	+	-	-	+	-	-
11		+	+	-	-	+	-	-
12		+	-	-	-	-	-	-
13		+	-	-	-	-	-	-
14		+	+	-	-	+	-	-
15		+	+	-	-	+	-	-
16		+	+	-	-	+	-	-
17		+	+	-	-	+	-	-
18		+	+	-	-	+	-	-
19		+	+	-	-	+	-	-
20		+	+	-	-	+	-	-
21		+	-	-	-	-	-	-
22		+	-	-	-	-	-	-
23		+	-	+	-	-	+	-
24		+	-	-	-	-	-	-
25		+	-	-	-	-	-	-
26		+	-	-	-	-	-	-
27		+	-	-	-	-	-	-
28		+	-	-	-	-	-	-
29		+	-	-	-	-	-	-
30	**Soybean Flour**	+	+	-	-	+	-	-

Table 5.8 (continued) : The results of the screening samples.

Sample No	Sample Type	Plant Specific PCR	35S Specific PCR	NOS Specific PCR	FMV Specific PCR	35S, FMV, NOS Specific Multiplex PCR		
						35S	NOS	FMV
31		+	-	-	-	-	-	-
32		+	-	-	-	-	-	-
33		+	-	-	-	-	-	-
34		+	-	-	-	-	-	-
35	**Soybean**	+	+	-	-	+	-	-
36	**Flour**	+	+	-	-	+	-	-
37		+	+	-	-	+	-	-
38		+	-	-	-	-	-	-
39		+	-	-	-	-	-	-
40		+	+	-	+	+	-	+
41		+	-	-	-	-	-	-
42		+	-	-	-	-	-	-
43		+	-	-	-	-	-	-
44		+	+	-	+	+	-	+
45	**Maize**	+	+	-	-	+	-	-
46		+	-	-	-	-	-	-
47		+	-	-	-	-	-	-
48		+	-	-	-	-	-	-
49		+	-	-	-	-	-	-
50		+	-	-	-	-	-	-
51		+	-	-	-	-	-	-
52		+	-	-	-	-	-	-
53		+	-	-	-	-	-	-
54		+	-	+	-	-	+	-
55		+	-	-	-	-	-	-
56		+	-	-	-	-	-	-
57		+	-	-	-	-	-	-
58		+	-	-	-	-	-	-
59		+	-	-	-	-	-	-
60		+	-	-	-	-	-	-
61		+	-	-	-	-	-	-
62		+	-	-	-	-	-	-
63		+	-	-	-	-	-	-
64	**Maize oil**	+	-	-	-	-	-	-

Table 5.8 (continued) : The results of the screening samples.

Sample No	Sample Type	Plant Specific PCR	35S Specific PCR	NOS Specific PCR	FMV Specific PCR	35S, FMV, NOS Specific Multiplex PCR		
						35S	NOS	FMV
65		+	-	-	-	-	-	-
66		+	-	-	-	-	-	-
67		+	-	-	-	-	-	-
68	**Maize oil**	+	-	-	-	-	-	-
69		+	-	-	-	-	-	-
70		+	-	-	-	-	-	-
71		+	-	-	-	-	-	-
72	**Tallow oil**	+	-	-	-	-	-	-
73		+	-	-	-	-	-	-
74		+	-	-	-	-	-	-
75		+	-	-	-	-	-	-
76		+	-	-	-	-	-	-
77		+	-	-	-	-	-	-
78		+	-	-	-	-	-	-
79	**Cat food**	+	-	-	-	-	-	-
80		+	-	-	-	-	-	-
81		+	-	-	-	-	-	-
82		+	-	-	-	-	-	-
83		+	-	-	-	-	-	-
84	**Dog food**	+	-	-	-	-	-	-
85		+	-	-	-	-	-	-
86		+	-	-	-	-	-	-
87		+	-	-	-	-	-	-
88		+	-	-	-	-	-	-
89		+	-	-	-	-	-	-
90		+	-	-	-	-	-	-
91		+	-	-	-	-	-	-
92		+	-	-	-	-	-	-
93		+	-	-	-	-	-	-
94	**Bread**	+	-	-	-	-	-	-
95		+	-	-	-	-	-	-
96	**Baklava**	+	-	-	-	-	-	-

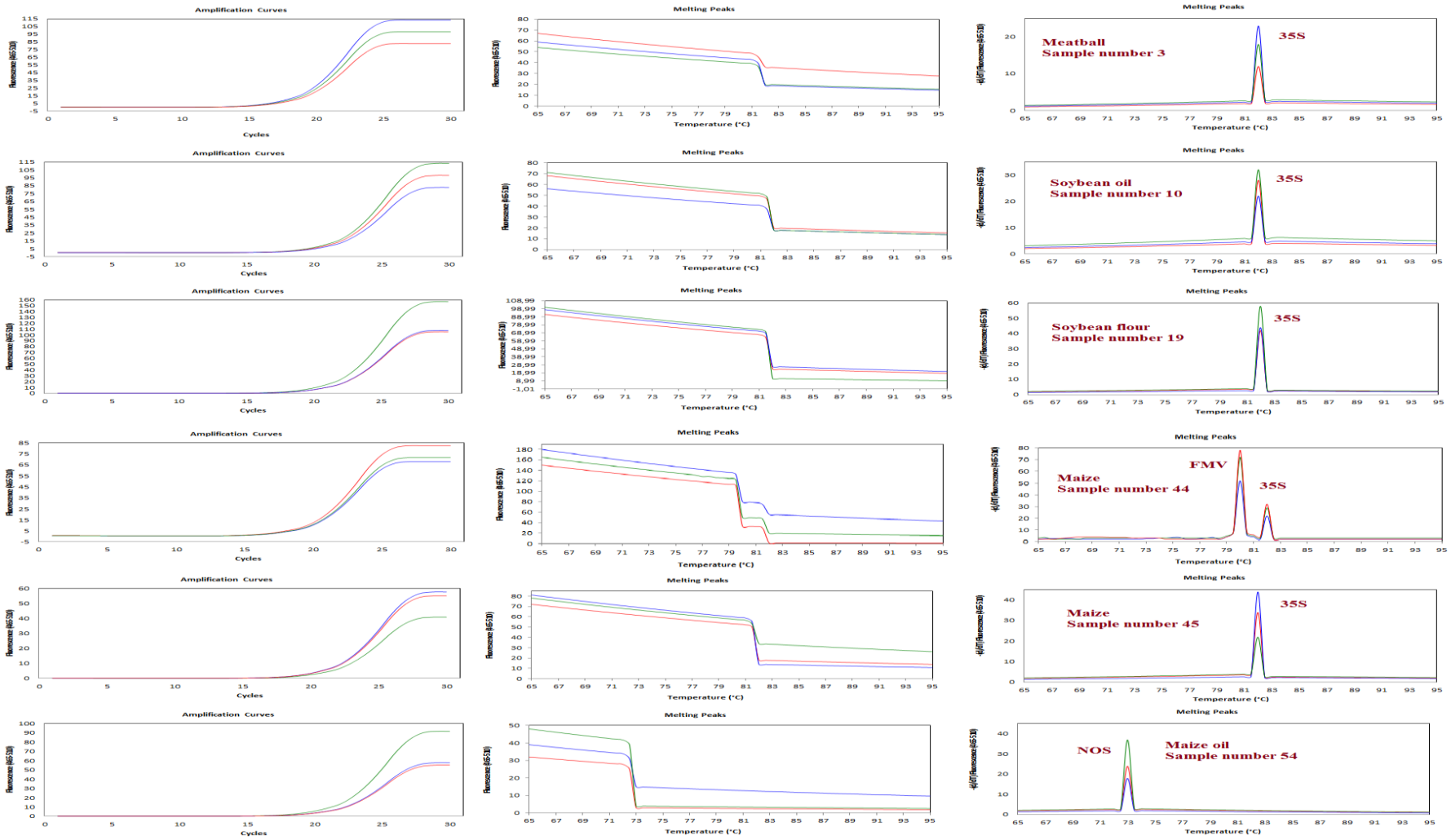


Figure 5.13 : The amplification curves (a, b, c, d, e, f, g), melting curves (g, h, i, j, k, l) and melt peaks (m, n, o, p, q, r) of one of the types of the screning samples (sample number 10, 19, 44, 45). First, second and third runs were shown in blue, red and green, respectively.

6. CONCLUSION

The current methodologies for QPCR based GMO screening are not time and cost effective. The main reasons behind these are the long incubation times and high costs of enzymes used in DNA extraction and the high costs of hydrolysis and hybridization probes used in the multiplex QPCR. The major outcome of this study was the development of a quick and low-cost QPCR-based system to qualitatively detect GM in the food products. This was achieved via an enzyme free DNA extraction methodology and a multiplex QPCR methodology using a single HRM dye. For the first time, this study introduced discrimination of the QPCR amplicons from the 35S, NOS and FMV elements based on the differences in their melting temperatures (T_m). The LODs of the methodology to detect 35S, NOS and FMV targets were in the desired ranges: 1 gene copies/gr food sample. The results also showed that all of the PCR amplicons were specific.

The expected T_m values corresponding to 35S, FMV and NOS targets were at 82, 80 and 73 ˚C, respectively. In the uniplex, binary and triplex QPCR trials of the reference samples, we found T_m values of each targets at the expected temperatures with deviation lower than 1 ˚C. The same T_m values were also obtained from the analysis of raw and processed foods that were already analyzed by accredited food laboratories. Our results were in 100% accordance with the results obtained by the accredited food control laboratories.

Screening for the GMO promoters or terminators is usually the first step for GMO analysis. Event specific qualitative and quantitative GMO analyses must subsequentially be carried on the GMO positive samples to ensure that the detected GMOs were not originated from the contaminations. This has a substantial importance in countries where the quantitative threshold levels were defined for labeling of the GM products.

6.1 Future Prospects

The developed methodology will be further validated by Turkey's oldest food control laboratory, Environmental Industrial Analysis Laboratory. We are also planning to develop an automated DNA isolation, PCR set-up and QPCR system and adapt our methodology to this system.

REFERENCES

Adugna, A., and Mesfin. T. (2008). Detection and quantification of genetically engineered crops. Journal of SAT Agricultural Research 6.

Al-Janabi, S.M., McClelland, M., Petersen, C., Sobral, B.W.S. (1994) Phylogenetic analysis of organellar DNA sequences in the Andropogoneae: Saccharinae. Theoretical and Applied Genetics 88: 933-944.

Allen, R.C., Rogelj, S., Cordova, S.E., Kieft, T.L. (2006). An immuno-PCR method for detecting Bacillus thuringiensis Cry1Ac toxin. J. Immunol. Methods 308, 109–115.

Andersen, C.B., Jensen, A.H., Berdal, K.G., Thorstensen, T., Tengs, T. (2006). Equal performance of TaqMan, MGB, molecular beacon, and SYBR green-based detection assays in detection and quantification of roundup ready Soybean. J. Agric. Food Chem. 54, 9658– 9663.

Anklam, E., Gadani, F., Heinze, F., Pijnenburg, H., Eede, G. V. D. (2002). Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. Eur Food Res Technol 214:3–26.

Anklam. E., Gadani. F., Heinze. P., Pijnenburg. H., Van den Eede. G. (2002). Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. European Food Research and Technology, 214: 3-2.

Babu, R.M., Sajeena, A., Seetharaman, K., Reddy, M.S. (2003). "Advances in genetically engineered (transgenic) plants in pest management- an overwiew" in Crop Protection 22: 1071-1086.

Bahrdt, C., Krech, AB., Wurz, A., Wulff, D. (2010). Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. Anal. Bioanal. Chem. 396, 2103–2112.

BATS report (2003). Genetically Modified (GM) Crops: molecular and regulatory details.

BATS Report (Agency for Biosafety Research and assessment of technology impact of swiss priority program biotechnology of swiss national science foundation). (2003). Foods derived from Genetically Modified Organisms and Detection Methods.

Beyer. P., Al-Babili S., Ye X., Lucca P., Schaub P., Welsch R., Potrykus I. (2002). Golden Rice: Introducing the Carotene Biosynthesis Pathway

into Rice Endosperm by Genetic Engineering to Defeat Vitamin A Deficiency. J. Nutr. 132: 506S–510S.

Broeders, S. R. M., Keersmaecker, S. C. J. D., Roosens N. H. C. (2012). How to Deal with the Upcoming Challenges in GMO Detection in Food and Feed. Journal of Biomedicine and Biotechnology.

Buh, G. M., Cankar, K., Zel, J., Gruden, K. (2008). Comparison of different real-time PCR chemistries and their suitability for detection and quantification of genetically modified organisms. BMC Biotechnol. 8, 26.

Cankar, K., Štebih, D., Dreo, T., Žel, J., Gruden, K. (2006). Critical points of DNA quantification by real-time PCR – effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. BMC Biotechnology 2006, 6:37.

Chelsea, S., Aude, B., Jean-Baptiste, B., Marcel, K., Gérard, P., Alain, P., & Ricroch, A. E. (2011). Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: a literature review. Food and Chemical Toxicology. 50, 1134–1148

Crickmore, N. (2011). Bacillus thuringiensis toxin nomenclature. Available at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/.

Demeke,T., Jenkins, G. (2009) Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. Anal Bioanal Chem.

Donohoe, G. G., Laaksonen, M., Pulkki, K., Rönnemaa, T., & Kairisto, V. (2000). Rapid single-tube screening of the C282Y hemochromatosis mutation by real-time multiplex allele-specific PCR without fluorescent probes. Clinical chemistry, 46(10), 1540-1547.

Dorak, M. T. (Ed.). (2007). Real-time PCR. Taylor & Francis.

Dörries, H. H., Remus, I., Grönewald, A., Grönewald, C., & Berghof-Jäger, K. (2010). Development of a qualitative, multiplex real-time PCR kit for screening of genetically modified organisms (GMOs). Analytical and bioanalytical chemistry, 396(6), 2043-2054.

Elsanhoty, R. M., Ramadan, M. F., & Jany, K. D. (2011). DNA extraction methods for detecting genetically modified foods: A comparative study. Food Chemistry, 126(4), 1883-1889.

Farid, E.A., (2002). Detection of genetically modified organisms in foods. Trends in Biotechnology. 20:215-223.

Griffiths, K., Partis, L., Croan, D., Wang, N., Emslie, K. (2003). Review of technologies for detecting genetically modified materials in commodities and food. Australian Government Analytical Laboratories.

Gudnason, H., Dufva, M., Bang, D. D., & Wolff, A. (2007). Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. Nucleic acids research, 35(19), e127.

Guo, J., Yang, L., Chen, L., Morisset, D., Li, X., Pan, L., Zhang, D. (2011). MPIC: One high-throughput analytical method for multiple DNA targets. Anal. Chem. 83, 1579–1586.

Herrmann, M. G., Durtschi, J. D., Wittwer, C. T., & Voelkerding, K. V. (2007). Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. Clinical chemistry, 53(8), 1544-1548.

Holst-Jensen, A., Rønning. S. B., Løvseth, A., Berdal, K.G. (2003). PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem 375 : 985–993.

ISO 21569:1-69 (2005). Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods"

James, C. (2012). Global Status of Commercialized Biotech/GM Crops: 2012. ISAAA Brief No. 43. ISAAA: Ithaca, NY.

Jha, A. (2012). Julian Ma: I'm growing antibodies in tobacco plants to help prevent HIV. The Guardian.

Kim, HY., Kim, JH, and Oh, MH. (2010). Regulation and detection methods for genetically modified foods in Korea. Pure Appl. Chem., Vol. 82, No. 1, pp. 129–137.

Kim, JH., Kim, SY., Lee, H., Kim, YR., Kim, HY. (2010). An event-specific DNA microarray to identify genetically modified organisms in processed foods. J. Agric. Food Chem. 58, 6018–6026.

Kumar, G. B. S., Ganapathi, T. R., Revathi, C. J., Srinivas L., Bapat V. A. (2005). "Expression of hepatitis B surface antigen in transgenic banana plants". Planta. 222, 484-493.

Leimanis, S., Hern´andez, M., Fern´andez, S., Boyer, F., Burns, M., Bruderer, S., Glouden, T., Harris, N., Kaeppeli, O., Philipp, P., Pla, M., Puigdom`enech, P., Vaitilingom, M., Bertheau, Y., Remacle, J. (2006). A microarray-based detection system for genetically modified (GM) food ingredients. Plant Mol. Biol. 61, 123–139.

Li, Q., Luan, G., Guo, Q., Liang, J. (2002). A new class of homogeneous nucleic acid probes based on specific displacement hybridization. Nucleic Acids Res. 30.

Liu, K. (1999). Biotech crops: products, properties and prospects. Food Technology, 53(5):42–9.

Mao, F., Leung, W. Y., & Xin, X. (2007). Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. BMC biotechnology, 7(1), 76.

Morisset, D., Dobnik, D., Hamels, S., Zel, J., Gruden, K. (2008). NAIMA: target amplification strategy allowing quantitative on-chip detection of GMOs. Nucleic Acids Res. 36, e118.

Nadal, A., Esteve, T., Pla, M. (2009). Multiplex polymerase chain reactioncapillary gel electrophoresis: a promising tool for GMO screeningassay for

simultaneous detection of five genetically modified cotton events and species. J. AOAC Int. 92, 765–772.

Nazarenko, I., Pires, R., Lowe, B., Obaidy, M., Rashtchian, A. (2002). Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. Nucleic Acids Res. 30, 2089–2095.

Newell, C. A. (2000). Plant Transformation Technology. Molecular Biotechnology. 16, 53-65.

Nollet, L. M. L. (2011). Safety Analysis of Foods of Animal Origin. Chapter 5.

Oliver, M. J. (2012). Plant gene containment. Y. Li (Ed.). Wiley-Blackwell.

Paine, J. A., Shipton, C. A., Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S. Y., Hinchliffe, E., Adams, J. L., Silverstone, A. L., Drake, R. (2005).Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat. Biotechnol. 23, 482-487.

Pan. L., Zhang. S., Yang. L., Broll. H., Tian. F., Zhang. D. (2007). Interlaboratory trial validation of an event-specific qualitative polymerase chain reaction-based detection method for genetically modified RT73 rapeseed. J AOAC Int 90:1639-1646.

Pauli, U., Liniger, M., Zimmermann, A. & Schrott, M. (2000). Extraction and amplification of DNA from 55 Foodstuffs in Mitt. Lebensm. Hyg. 91: 491-501.

Querci, M., Van den Eede, G., Jermini, M. (2006). "The Analysis of Food Samples for the Presence of Genetically Modified Organisms" European Comission Joint Research Center Manuel, Session 1-12.

Randhawa, G. J., Chhabra, R., Singh, M. (2009). Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for the screening of genetically modified crops. J. Agric. Food Chem. 57, 5167–5172.

Redenbaugh, Keith, Bill Hiatt, Belinda Martineau, Matthew Kramer, Ray Sheehy, Rick Sanders, Cathy Houck and Don Emlay (1992), Safety Assessment of Genetically Engineered Fruits and Vegetables: A Case Study of the Flavr Savr Tomato, CRC Press. p. 288

Reiting. R., Broll. H., Waiblinger. H.-U., Grohmann. L. (2007). Collaborative Study of a T-nos Real-Time PCR Method for Screening of Genetically Modified Organisms in Food Products. J. Verbr. Lebensm. 2:116-121.

Ronald, P. (2011). Sustainable Agriculture and Global Food Security. Genetics, 188: 11–20.

Royal Society, T (2009). Reaping the Benefits: Science and the Sustainable Intensification of Global Agriculture. The Royal Society, London.

Salvi, S., D'Orso, F., Morelli, G. (2008). Detection and quantification of genetically modified organisms using very short, locked nucleic acid TaqMan probes. J. Agric. Food Chem. 56, 4320–4327.

Séralini, G. E., Clair, E., Mesnage, R., Gress, S., Defarge, N., Malatesta, M., and de Vendômois, J. S. (2012). Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food and Chemical Toxicology. 50, 4221–4231.

Shim, Y.Y., Shin, W.S., Moon, G.S., Kim, K.H. (2007). Quantitative analysis of phosphinothricin-N-acetyltransferase in genetically modified herbicide tolerant pepper by an enzyme-linked immunosorbent assay. J. Microbiol. Biotechnol. 17, 681–684.

Tavernier, I., Van Bockstaele, E. and De Loose, M. (2004). Cloned plasmid DNA fragments as calibrators for controlling GMOs: Different real-time duplex quantitative PCR methods. Analytical and Bionalytical. Chemistry 378(5): 1198-1207.

Taverniers, I., Papazova, N., Allnutt, T., Baumler, S., Bertheau, Y., Esteve, T. and Zhang, D. (2013). Harmonised Reference Genes and PCR Assays for GMO Quantification. Genetically Modified and Non-Genetically Modified Food Supply Chains: Co-Existence and Traceability, 273-292.

Thelwell, N., Millington, S., Solinas, A., Booth, J., Brown, T. (2000). Mode of action and application of Scorpion primers to mutation detection. Nucleic Acids Res. 28, 3752–3761.

Tripathi, L. (2005). Techniques for detecting genetically modified crops and products. African Journal of Biotechnology, Vol. 4 (13), pp. 1472-1479.

Tung Nguyen, C. T., Son, R., Raha, A. R., Lai, O. M. and Clemente Michael, W. V. L. (2009). Comparison of DNA extraction efficiencies using various methods for the detection of genetically modified organisms (GMOs). International Food Research Journal, 16:21-30.

Van, D. G., Van, B. R., Bleeker-Marcelis, H., Van, B. I., Adan, A.J., Jhakie, S., Hessing, M. (2002). Detection of genetically modified organisms in foods by PCR- and DNA-based techniques: bridging the methods. Journal of AOAC International, 85, 787-791.

Viljoen, C.D., Dajee, B.K., and Botha, G.M. (2006). Detection of GMO in food products in South Africa: Implications of GMO labelling. African Journal of Biotechnology Vol. 5 (2), pp. 73-82.

Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., Little, S. (1999). Detection of PCR products using self-probing amplicons and fluorescence. Nat. Biotechnol. 17, 804–807.

Wilkinson, J.Q. (1997) Biotech plants: from lab bench to supermarket shelf. Food Technol , 51(12):37–42.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplifi- cation. Applied and Environment Microbiology, 63, 3741–3751.

Xu, J., Zhu, S., Miao, H., Huang, W., Qiu, M., Huang, Y., Fu, X., Li, Y. (2007). Event-specific detection of seven genetically modified soybean and maizes using multiplex-PCR coupled with oligonucleotide microarray. J. Agric. Food Chem. 55, 5575–5579.

Yang, DY., Eng, B., Waye, JS., Dudar, JC., Saunders, SR. (1998). Technical Note: Improved DNA Extraction from Ancient Bones Using Silica-Based Spin Columns. American Journal of Physical Anthropology 105, 539-543.

Yao, Q., Cong, L., Chang, J. L., Li, K. X., Yang, G. X. and He, G. Y. (2006) Low copy number gene transfer and stable expression in a commercial wheat cultivar via particle bombardment. Journal of Experimental Botany 57(14):3737-3746.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P., Potrykus, I. (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carentonoid-free) rice endosperm. Science. 287, 03-305.

Žel, J., Milavec, M., Morisset, D., Plan, D., Van den Eede, G., & Gruden, K. (2012). How to reliably test for GMOs (pp. 1-95). Springer US.

APPENDICES

APPENDIX A: QPCR results and Sequence analysis results of NOS, FMV, 35S and PLANT regions.

APPENDİX A

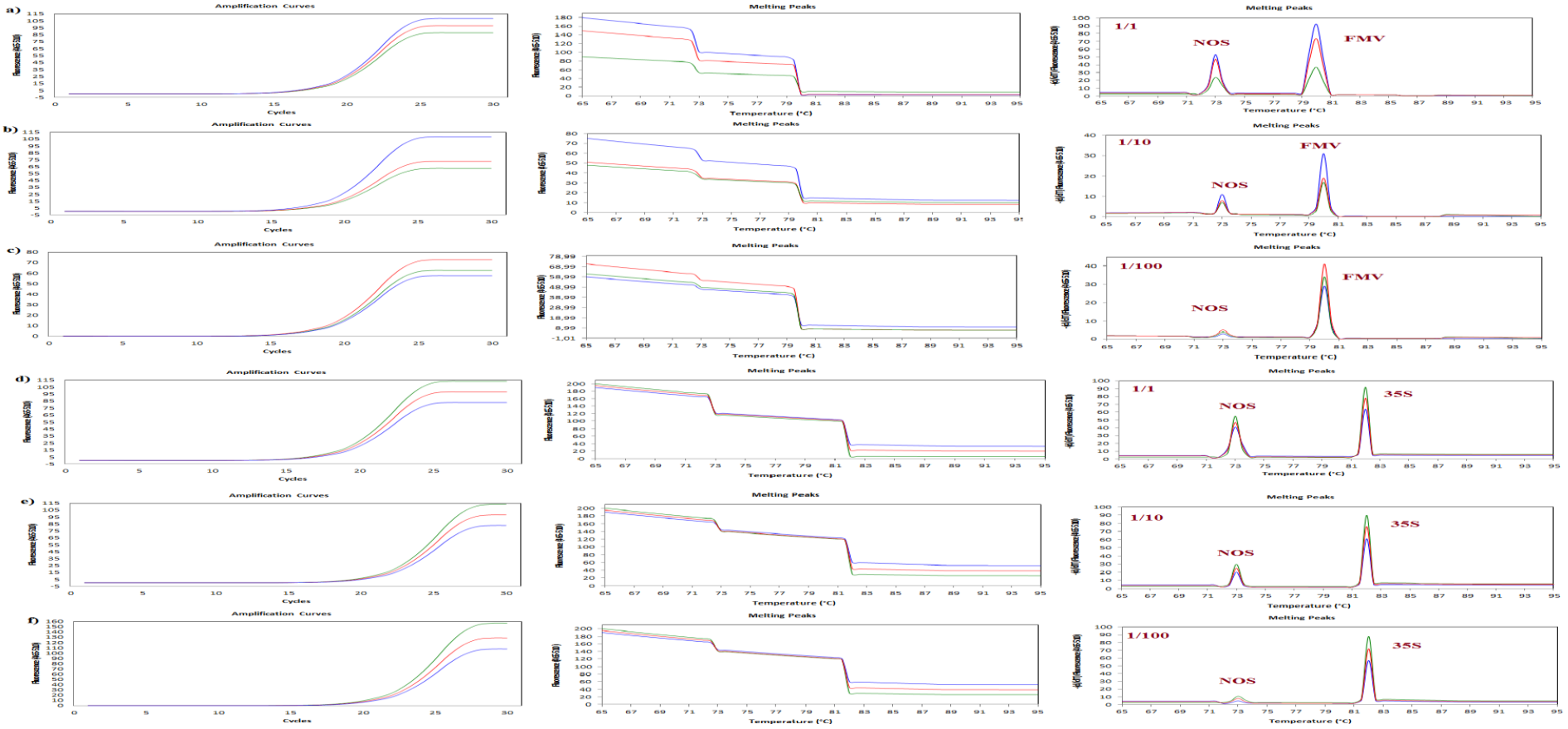


Figure A.1 : QPCR results of NOS, FMV and 35S when mixed at different ratios (1/1, 1/10, 1/100).

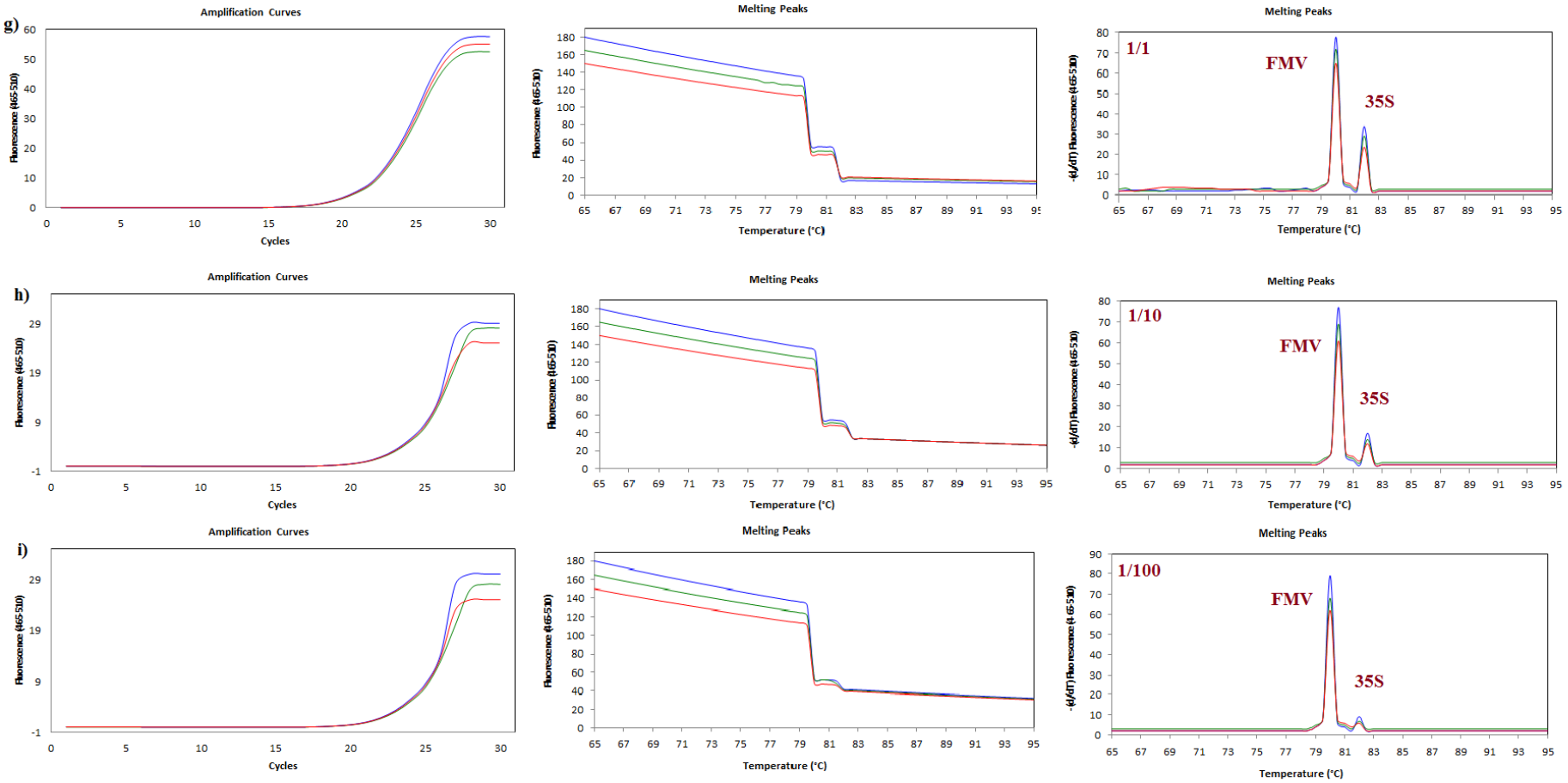
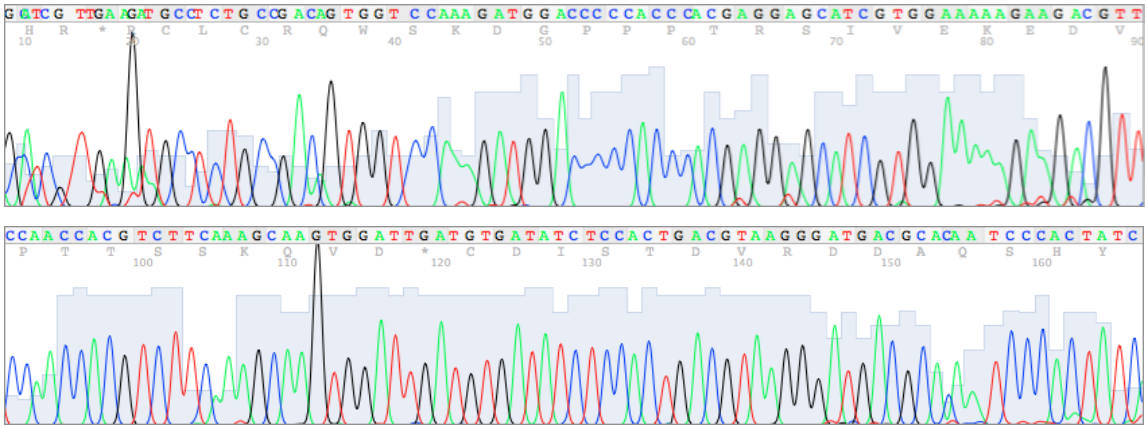
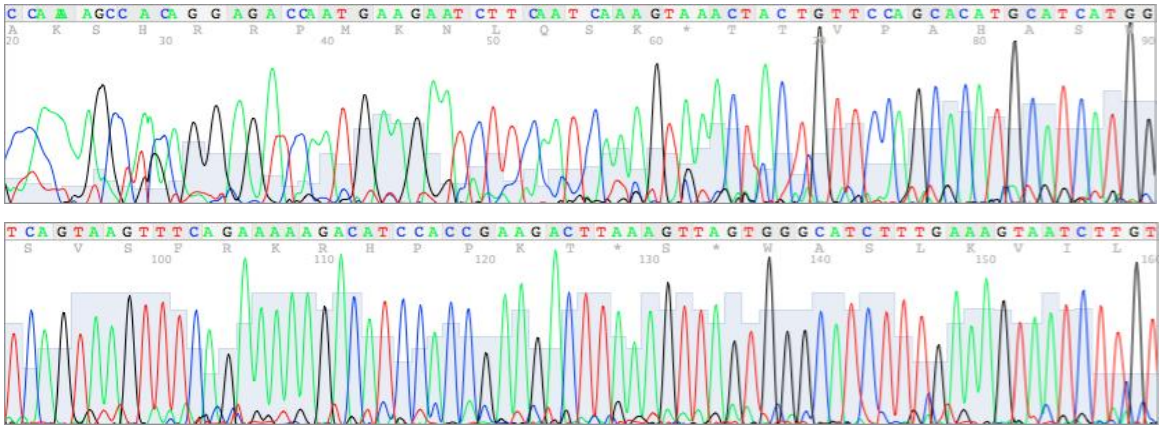


Figure A.1 (continued) : QPCR results of NOS, FMV and 35S when mixed at different ratios (1/1, 1/10, 1/100).

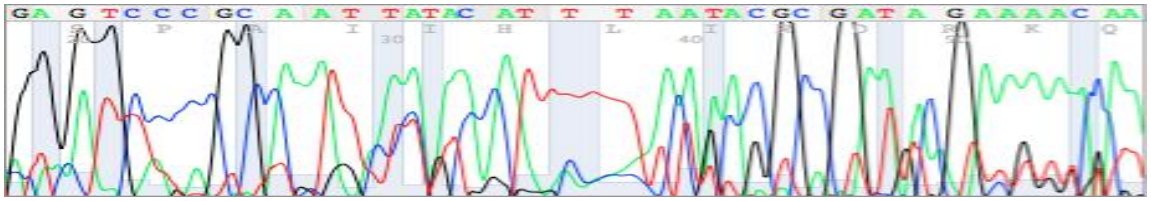
a)



b)



c)



d)

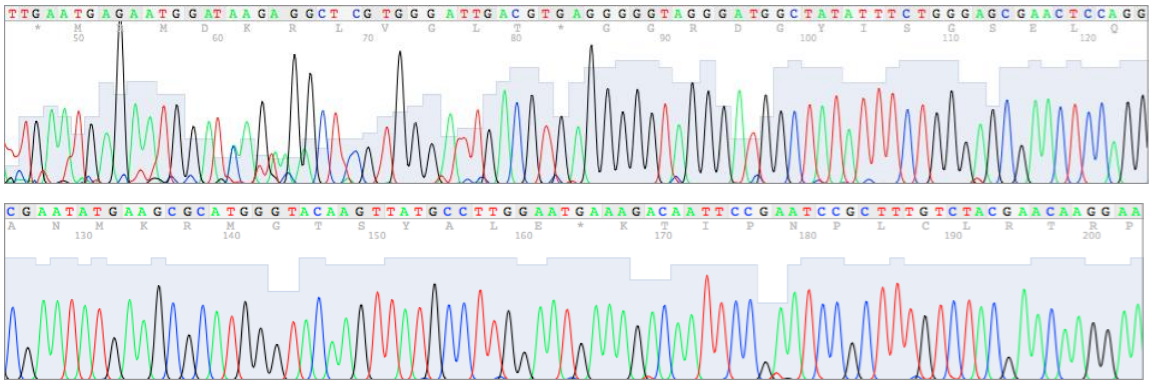
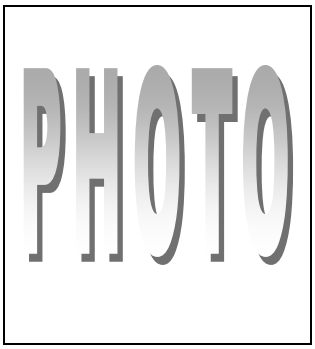


Figure A.2 : The Chromatogram result of obtained 35S (a), FMV (b), NOS (c), Plant (d) sequences.

CURRICULUM VITAE



Name Surname: Deniz Gülbin Tan

Place and Date of Birth: Izmir, 06.04.1987

Address: Murat Reis mah. Topal sok. Bülbül apt. No:17-19 D:6 ÜSKÜDAR/ ISTANBUL

E-Mail: denizgtan@gmail.com

B.Sc.: Istanbul University

M.Sc.: Istanbul Technical University

Professional Experience and Rewards:

List of Publications and Patents:

PUBLICATIONS/PRESENTATIONS ON THE THESIS

- Tan, D. G., Kolukırık, M., Dinler, G. 2013: Development of a Fast QPCR-Based Method for GMO Detection. *European Biotechnology Congress*, March 16-18, 2013 Bratislava, Slovakia.