SCREENING FOR GENETICALLY MODIFIED TOMATOES & TOMATO SEEDS AND IDENTIFICATION OF CRY1AC AND SAM-K SPECIFIC MODIFICATIONS USING GENE AND CONSTRUCT SPECIFIC PCR

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

BY

ESRA UÇKUN

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

SEPTEMBER 2007
SCREENING FOR GENETICALLY MODIFIED TOMATOES &
TOMATO SEEDS AND IDENTIFICATION OF CRY1AC AND SAM-K
SPECIFIC MODIFICATIONS USING GENE AND CONSTRUCT
SPECIFIC PCR

submitted by ESRA UÇKUN in partial fulfillment of the requirements for the
degree of Master of Science in Biotechnology Department, Middle East
Technical University by,

Prof. Dr. Canan Özgen
Dean, Graduate School of Natural and Applied Sciences

Prof. Dr. Fatih Yıldız
Head of Department, Biotechnology Dept., METU

Assoc. Prof. Dr. G.Cândan Gürakan
Supervisor, Food Engineering Dept., METU

Prof. Dr. Mükerrer Kaya
Co-Supervisor, Food Engineering Dept., Atatürk Univ.

Examining Committee Members:

Prof. Dr. Meral Yücel
Biology Dept., METU

Assoc. Prof. Dr. G.Cândan Gürakan
Food Engineering Dept., METU

Prof. Dr. Mükerrer Kaya
Food Engineering Dept., Atatürk University

Prof. Dr. Haluk Hamamcı
Food Engineering Dept., METU

Assoc. Prof. Dr. Sertaç Önde
Biology Dept., METU

Date: 07.09.2007
I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Esra Uçkun

Signature: 

iii
ABSTRACT

SCREENING FOR GENETICALLY MODIFIED TOMATOES & TOMATO SEEDS AND IDENTIFICATION OF CRY1AC AND SAM-K SPECIFIC MODIFICATIONS USING GENE AND CONSTRUCT SPECIFIC PCR

Uçkun, Esra
M.Sc., Department of Biotechnology
Supervisor: Ass.Prof. Dr. G.Candan Gürakan
Co-Supervisor: Prof. Dr. Mükberrem Kaya

September 2007, 142 pages

This study was carried out to analyze tomato samples and tomato seeds, purchased from different food markets of Turkey randomly, for the presence of genetic modification by using PCR method as it allows more specific detection. The DNAs of collected samples were isolated according to CTAB DNA extraction protocol and also with extraction kits. Screening tests of tomatoes were done by targeting 35S promoter, NOS terminator and NptII kanamycin resistance gene with eight different primer sets. Real time PCR is used to confirm 35S and NOS positives results obtained from conventional PCR.

In this study, it was observed that 14 out of 35 seed samples, and 14 out of 40 fresh tomato samples which were screened had at least one transgenic element of 35S
promoter, NOS terminator and NPTII kanamycin resistance gene indicating the possible presence of genetic modifications.

After screening, gene specific studies were carried out for PG, sam-k indicating F type ripening delayed tomato and the 35 1 N lines respectively and cry1Ac genes inserted in 5345-1 insect resistant tomato line. PG and sam-k specific primers were not amplified in any of the samples investigated whereas 18 out of 75 samples were cry1Ac positive and 1 out of 75 samples was sam-k positive. Positives were confirmed by sequence analysis.

Additionally, construct specific primers specific to 5345-1 and 35 1 N lines were designed. PCR amplicons indicate the existence of the construct sequence. In order to verify the results, PCR products were sent to sequence analysis.

**Key words:** Genetically modified organisms, GMO detection, insect resistant tomato, PCR, real time PCR, construct-specific PCR.
ÖZ

DOMATES VE DOMATES TOHUMLARINDA GENETİK MODİFİKASYONUN TESPİTİ VE CRY1AC VE SAMK GENLERİNİN GEN VE KONSTRAKT SPESİFİK PZR YÖNTEMLERİ İLE SAPTANMASI

Uçkun, Esra
Yüksek Lisans, Biyoteknoloji Bölümü
Tez yöneticisi: Doç. Dr. G.Candan Gürakan
 Ortak tez yöneticisi: Prof. Dr. Mükkerrem Kaya

Eylül 2007, 142 sayfa

Bu tez çalışması Türkiye'nin çeşitli illerinden alınan domates ve domates tohum örneklerinde genetiği modifiye gıdaların tespitinde en güvenilir metod olan PZR kullanılarak genetik modifikasyonun tespiti için gerçekleştirilmiştir. Toplanan örneklerin DNAları CTAB ekstraksyon protokolü ve ekstraksiyon kitleri kullanılarak sağlanmıştır. Domateslerin nitel taraması için 35S, NOS ve NPTII bölgeleri hedef alınarak 8 farklı primer seti kullanılmıştır. Real time PZR, konvansiyonel PZR ile 35S ve NOS pozitif bulunan örnekleri kontrol etmek amacıyla kullanılmıştır.
Bu çalışmada, 35 tohum örnekinin 14'ünde ve 40 domates örnekinin 14'ünde genetik modifikasyonun varlığını işaret eden 35S promotör, NOS terminatör, kanamisin direnç geninin en az biri tespit edilmiştir.

Tarama sonrasında, gen spesifik çalışmaları F tipi oltunlaşması geçikleştirmiş GM domasteste bulunan poligalakturonaz geni , 35 1 N GM domates türünde bulunan sam-k ve böcek dirençli 5345-1 GM domates türünde bulunan cry1Ac genleri hedef alınarak yapılmıştır. PG ve sam-k genlerine özgü primerler hiçbir örnekte yükseltgenmezken, toplam 18 örnekte cry1Ac geni ve 1 örnekte sam-k geni saptanmış ve sekanslanarak doğrulanmıştır.

Ayrıca, 5345-1 ve 35 1 N türü konstraktlara özgü primerler dizayn edilmiştir. PZR sonuçları konstraktın varlığına işaret etmektedir. Konstraktın varlığının emin olmak amacıyla PZR ürünleri dizi analizine gönderilmiştir.

**Anahtar kelimeler:** böcek dirençli domates, eş zamanlı PZR, GDO tespiti, genetiği modifiye organizmalar, PZR.
TO MY FAMILY; MEHMET, HAVVA, SEDA
ACKNOWLEDGEMENTS

I would like to express my greatest thanks to my supervisor Assoc. Prof. G. Candan Gürakan for her guidance, invaluable advices, help, friendship and patient insight throughout the research.

I would like to express my gratitude to my co-supervisor Prof. Dr. Mükerrerem Kaya for his advices and comments. I should also mention his kind attitude towards me when I was in Erzurum.

I would like to thank to Prof. Dr. Chris Bowler and Gökhan Duruksu for the positive tomato seeds samples which I used during this study and thank to Prof. Dr. Meral Yucel to support the plasmids and also thank to BGSC to send me cry1Ac positive E.coli and B. thuringiensis.

I would also like to thank the members of thesis examining committee; Prof. Dr. Meral Yücel, Prof. Dr. Mükerrerem Kaya, Assoc. Prof. Dr. Sertaç Önde and Prof. Dr. Haluk Hamamcı for their suggestions and comments.

I can not express my gratitude to all my lab. colleagues and Genetic lab group just with words, but if I have to I would like to thank Gökhan Duruksu, Dr. Remziye Yılmaz, Dr. Evren Koban, Abdullah Tarakçıoğlu for their guidance and patience to my endless questions, Aysun Cebeci Aydın, Humen Jabbarifarthoud for their support and patience, Rahşan Ivgin Tunca for her friendship and help during my PCR experiments, Özlem Erçin, for her help and kindness during my study especially during transformation experiments, Erhan Astarci for his help during DNA
isolation, Bengü Öztürk, Neslihan Altay, Sevgi Özen, Çiğdem Moral for their friendship and kindness.
I would like to thank to Bahtiyar Can, representative of Esmed, for providing DNA extraction kit and Hakan Moral for his technical supports, patience and friendship especially when I became crazy with the computer, Dr. Christopher Halldeman, Dr. Edna Levi for their valuable advices. Also I want to thank all the people who collected tomatoes from different parts of Turkey and the world.

Finally but not leastly, I am greatly indebted to my angels Fadime Kara and Derya Sevinç for their great help, support, patience, kindness, friendship, presence and everything. They are such great persons and I am very lucky to be near them.

Finally, I am grateful to my parents Mehmet, Havva and my sister Seda, thanks for your love, patience, endless support and confidence in me.
# TABLE OF CONTENTS

PLAGIARISM ........................................................................................................ iii

ABSTRACT ........................................................................................................... iv

ÖZ ......................................................................................................................... vi

ACKNOWLEDGEMENTS ..................................................................................... ix

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

LIST OF TABLES ................................................................................................. xvii

LIST OF FIGURES .............................................................................................. xix

LIST OF ABBREVIATIONS .................................................................................. xxii

CHAPTER

1- INTRODUCTION ........................................................................................... 1

1.1 Genetically Modified Organisms (GMO) .................................................... 1

1.2 GMO Detection ........................................................................................... 3

1.2.1 Legislation of Genetically Modified Foods (GMFs) .............................. 3

1.2.2 Regulation in EU ................................................................................... 5

1.2.3 Regulation in USA ................................................................................. 6

1.2.4 Regulation in Japan ................................................................................ 6

1.2.5 Regulation in China ................................................................................ 6
2.2 Reference Materials .......................................................... 28

2.2.1 DNA Isolation from Fresh Tomato Samples ....................... 29

2.2.2 DNA Isolation from Dry Tissue ........................................... 30

2.2.3 Plasmid DNA Isolation ..................................................... 31

2.3 Determining the DNA Purity .................................................. 32

2.4 Primer Design ................................................................. 33

2.5 Polymerase Chain Reaction (PCR) ......................................... 37

2.6 Amplification of Target Genes by PCR ................................... 38

2.6.1 Control PCR ................................................................. 38

2.6.2 Screening PCRs ............................................................. 39

   2.6.2.1 35S Promoter Specific PCR ......................................... 39

   2.6.2.2 NOS Terminator Specific PCR ...................................... 41

   2.6.2.3 NPTII Specific PCR .................................................... 42

2.6.3 Identification PCRs (Gene Specific Target) ......................... 43

2.6.4 Construct Specific PCR ................................................... 46

   2.6.4.1 Construct Specific PCR System for 5345-1 Line .................. 46

   2.6.4.2 Construct Specific PCR System for 35 1 N Line ............... 46
2.7 Visualization on Agarose Gels ............................................. 48
2.8 Real-Time PCR ........................................................................ 48
3-RESULTS AND DISCUSSION .................................................. 50
3.1 DNA Isolation for GMO Detection Analysis ............................ 50
3.2 PCR Amplifications ................................................................. 54
  3.2.1 Patatin Specific Control PCR System ................................. 54
  3.2.2 Screening PCR Systems for the Detection of GM Tomatoes 55
    3.2.2.1 CaMV-35S Promoter Specific PCR Systems .................. 56
    3.2.2.2. NOS Terminator Specific PCR Systems ....................... 62
    3.2.2.3 NPTII Gene Specific PCR ............................................ 65
3.2.3 Identification PCR Systems to Ripening Delayed Tomatoes.... 70
  3.2.3.1 Identification of Sam-k gene ......................................... 70
  3.2.3.2 Identification of PG Gene ............................................. 71
  3.2.3.3 Identification PCR Systems Specific to Insect Resistant Tomatoes .................................................. 72
3.2.4 Construct specific PCR Systems (Gene Specific Target Systems) .......................................................... 76
  3.2.4.1 Construct specific PCR Systems Specific to 5345-1 line .... 76
  3.2.4.2 Construct specific PCR Systems Specific to 351 N line .... 78
APPENDIX M ................................................................. 132

APPENDIX N ................................................................. 138
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Approved GM tomatoes</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>Primers used for screening studies</td>
<td>36</td>
</tr>
<tr>
<td>2.2</td>
<td>Primers used for identification studies</td>
<td>37</td>
</tr>
<tr>
<td>2.3</td>
<td>PCR conditions for patatin</td>
<td>39</td>
</tr>
<tr>
<td>2.4</td>
<td>PCR conditions for P-35S using 35SF1/R2 and p35Saf1u/p35S-ar1 primer sets</td>
<td>40</td>
</tr>
<tr>
<td>2.5</td>
<td>PCR conditions for 35S Promoter using 35S EU F/R Primer set</td>
<td>40</td>
</tr>
<tr>
<td>2.6</td>
<td>PCR conditions for NOS terminator using NOSf1/r2 Primer set</td>
<td>41</td>
</tr>
<tr>
<td>2.7</td>
<td>PCR conditions for NOS terminator using NOS 207f/NOS 207r primer set</td>
<td>42</td>
</tr>
<tr>
<td>2.8</td>
<td>PCR conditions for NPTII gene region using knF/R, KAN411F/R primer sets</td>
<td>43</td>
</tr>
<tr>
<td>2.9</td>
<td>PCR conditions for NPTII region using TN5 fw/rv primer set</td>
<td>43</td>
</tr>
<tr>
<td>2.10</td>
<td>PCR conditions for PG gene region using PG34L/PG34R primer set</td>
<td>45</td>
</tr>
<tr>
<td>2.11</td>
<td>PCR conditions for sam-k and cry1Ac gene regions using sam3f/5f and cry329f/r primer sets respectively</td>
<td>45</td>
</tr>
<tr>
<td>2.12</td>
<td>Construct specific PCR conditions using cscryf/r and sameuf/r primer sets</td>
<td>47</td>
</tr>
</tbody>
</table>
Table 2.13 Real-time PCR conditions for 35S promoter and NOS terminator regions.................................................. 49
Table 3.1 Concentration of seed samples DNAs according to the spectrophotometer and their cultivar types................................................................. 52
Table 3.2 Concentration of fresh tomato samples DNAs ...................... 53
Table 3.3 Sequences producing significant alignments with 35S promoter specific PCR results using P35S-afl1u/P35Sar1 primer set ..................................... 59
Table 3.4 Sequences producing significant alignments with 35S promoter specific PCR results using 35SEUF/R primer set .............................................. 61
Table 3.5 Sequences producing significant alignments with kanamycin resistance gene specific PCR products using kanF/R primer set ......................... 67
Table 3.6 Sequences producing significant alignments with cry1Ac gene specific PCR products using cry3Z3f/R primer set ........................................ 75
Table 3.7 Screening and identification PCR results of seed samples............ 88
Table 3.8 Screening and identification PCR results of fresh samples............ 90

xviii
LIST OF FIGURES

FIGURES

Figure 1.1 Global area of transgenic crops.............................................................. 2
Figure 1.2 The countries that Turkey import tomato seeds and the amount (Kg) between the years 2003-2005................................................................. 10
Figure 1.3 Genetic construct of insect resistant tomato line 5345-1............. 17
Figure 1.4. Scheme of qualitative PCR systems....................................................... 20
Figure 2.1 Some of the collected fresh tomato samples................................. 27
Figure 2.2 Some of the collected seed samples ............................................... 28
Figure 2.3 Genetic construct of ripening delayed tomato line 35 1 N........ 47
Figure 3.1 Patatin specific PCR results................................................................. 55
Figure 3.2 35S Promoter specific positive PCR results by using 35Sl/r primer set................................................................. 57
Figure 3.3 35S Promoter specific positive PCR results by using p35Saflu/p35Sar1 primer set ................................................................. 58
Figure 3.4 35S Promoter specific positive PCR results by using 35S EUF/R primer set................................................................. 59
Figure 3.5 NOS terminator specific positive PCR results using nosf/r primer set................................................................. 63
Figure 3.6 NOS terminator specific positive PCR results for seed samples using NOS207f/r primer set ................................................................. 64
Figure 3.7 NOS terminator specific positive PCR results for fresh samples using NOS207f/r primer set ................................................................. 64
Figure 3.8 ClustalW analysis result of the obtained Nos terminator sequence using NOS207f/R primer set and the U12540 Pbin NOS terminator sequence ................................................................. 65
Figure 3.9 NPTII specific positive PCR results using kanfr primer set .... 67
Figure 3.10 NPTII specific positive PCR results using kan411f/r primer set ........................................................................................................ 68
Figure 3.11 Clustal W analysis result of the obtained NPTII sequence using KAN411F/R primer set and the target AF274974 sequence .... 69
Figure 3.12 NPTII specific positive PCR results using TN5F/R primer set 70
Figure 3.13 Sam-k specific PCR results using sam3f/5f primer set ........ 71
Figure 3.14 PG gene-specific PCR results using PG34F/R primer set ..... 72
Figure 3.15 Cry1Ac gene-specific positive PCR results using cry323f/r primer set ...................................................................................... 74
Figure 3.16 Construct-specific PCR results specific to 5345-1 line using crycfs/crycscr primer set ........................................................................ 77
Figure 3.17 Construct-specific PCR results specific to 35 1 N line using sameuf/sameur primer set ...................................................................... 78
Figure 3.18 Clustal W analysis result of the obtained construct specific sam-k- nos junction sequence using sameuf/sameur primer set .......... 79
Figure 3.19 35S Promoter specific real-time PCR results of positive seed samples .......................................................................................... 80

xx
LIST OF ABBREVIATIONS

Acc: Aminocyclopropane carboxylate synthase

ACC: 1-aminocyclopropane 1-carboxylic acid

accd: 1-aminocyclopropane 1-carboxylic acid deaminase

AFLP: Amplified Fragment Length Polymorphism

APHIS: The Animal and Plant Health Inspection Service

μg : Microgram

μl : Microliter

BLAST : The Basic Local Alignment Search Tool

BGSC: Bacillus Genetic Stock Center

BgVV: Federal Institute for Health Protection of Consumers

bp : base pair

Bt: Bacillus thuringiensis
CaMV: Cauliflower mosaic virus

CBP: Cartagena Biosafety Protocol

Cs: Construct-specific

CTAB: cetyltrimethylammonium-bromide

Cry proteins: Crystal endotoxin proteins

DNA: Deoxyribonucleic acid

ds : double stranded

EC: European Commission

ECB: European Corn Borer

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

EMBL: European Molecular Biology Laboratory

EPA: Environmental Protection Agency

EtBr: Ethidium Bromide
EU: European Union

FDA: Food and Drug Administration

GMO: Genetically Modified Organisms

GMF: Genetically Modified Foods

GM: Genetically Modified

ICP: Insecticidal crystal protein

JRC: Joint Research Center

Kan: Kanamycin

kb: kilobase

LB medium: Luria Bertoni medium

LDR: ligation detection reaction

MAFF: The Ministry of Agriculture, Forestry and Fisheries

MHLW: The Ministry of Health, Labor and Welfare

min: minute
mM: Milimolar

mRNA: Messenger RNA
NCBI: National Centre for Biotechnology Information

ng: Nanogram

NOS: Nopaline synthase

NPTII: Neomycin phosphotransferase Type II

PCR: Polymerase Chain Reaction

PG: polygalacturonase

pmol: Pico mole

PVP: Polyvinylpyrrolidone

PZR: Polimeraz zincir reaksiyonu

QC-PCR: Quantitative Competitive – Polymerase Chain Reaction

rDNA: recombinant DNA

Rpm: Rotation per minute

RT-PCR: Real Time-Polymerase Chain Reaction
sam-k: S-adenosylmethionine hydrolase encoding gene

USDA: US Department of Agriculture
UV: Ultra Violet

v/v: volume/volume

w/v: weight/volume
CHAPTER 1

INTRODUCTION

1.1. Genetically Modified Organism (GMO)

Modern biotechnological techniques have rapidly expanded the horizons of plant breeding and crop improvement. Conventional breeding techniques rely on the random rearrangement of existing genes between two closely related parent plants. Genetic engineering has opened up new possibilities by allowing the transfer of individual, known genes, even from completely unrelated organisms such as fungi or bacteria.

Genetically Modified Organisms (GMOs) can be defined as organisms in which the genetic material (DNA) has been changed in a way that does not occur under natural conditions through cross-breeding or natural recombination (Article 2 of the EU Directive on the Deliberate Release into the Environment of Genetically Modified Organisms (2001/18/EG)).

The addition of foreign genes has often been used in plants to produce novel proteins that confer pest and disease tolerance and, more recently, to improve the chemical profile of the processed product (Anklam et al, 2000, Gachet et al., 1999).

The first genetically modified bacteria were produced in the early 1970s using recombinant DNA technology, and the first GM plant was produced in 1983. By the late 1980s, genetically modified (GM) crops which are virus resistant tobacco and
tomato were on sale in China, but they did not become widespread until 1994. The early examples of GM crops were delayed ripening "Flavr Savr®" tomato; insect-resistant corn that was introduced in 1995 and herbicide tolerant soybean that was introduced in 1996 (Hails & Kinderlerer, 2003).

Today, many millions of hectares of commercially produced transgenic crops such as, soybean, maize, cotton and canola has been grown annually in a number of countries, the countries with the largest GM cultivations are the USA, Argentina, Brazil, Canada, India and China. Total global area of crops reached 102 million hectares in 2006. Half of all GM cultivations worldwide are located in the USA. 10.3 million farmers in 22 countries grew GM soy, maize, rape and cotton. GM papaya, alfalfa, squash and rice are cultivated on smaller areas. (ISAAA Briefs No 35-2006., http://www.isaaa.org/resources/publications/briefs/35/executive summary/default.html, 2007).

![Figure 1.1 Global Area of Transgenic Crops (1996 to 2006), (ISAAA Briefs No 35-2006)]
1.2. GMO Detection

By the time GM crops began to find more place in supermarket shelves as food, detection and labelling of these crops gain much more importance. A number of GMOs have been approved for human consumption but concerns over safety persist in the public. Allergenicity and toxicity, which are caused by proteins, are the major concerns (Kuiper & Kleter, 2003). Moreover, depending on the region of the world, people often have different attitudes to food. In addition to concerns on safety and nutritional value, food often has societal and historical connotations, and in some instances may have religious importance. Genetically modified foods can evoke a negative response among consumers, especially in the absence of good communication on risk assessment efforts and cost/benefit evaluations.

In order to improve a positive impact on consumers, it is necessary to give true and satisfactory information. Regulations on GM foods and labelling are inevitable to provide confidence among consumers.

Moreover, labelling of genetically modified crops may provide an opportunity to the consumers whether they prefer to buy a GM food or not. Since highly controversial techniques are employed during GM crop development and the presence of data regarding negative effects of GMOs on human health as well as on the environment, the industry might benefit from true and informative labeling which most certainly improve public perception.

1.2.1 Legislation of Genetically Modified Foods (GMFs)

Virtually all GM crops assessed for safety to date have both food and feed use. As the greatest concerns have related to human exposure, not surprisingly the emphasis
of most regulatory authorities has been placed on food use. Safety for animals, while not ignored, has been a lesser concern and generally, and not unreasonably, been assumed to the follow automatically from the food safety assessment (Aumaitre et al., 2002).

Regulation of GMFs varies in different governments. In some countries GM foods are not yet regulated. Countries which have legislation in place focus primarily on assessment of risks for consumer health. Countries which have provisions for GM foods usually also regulate GMOs in general, taking into account health and environmental risks, as well as control- and trade-related issues such as potential testing and labelling regimes. In view of the dynamics of the debate on GM foods, legislation is likely to continue to evolve.

There are not many specific international regulatory systems however, several international organizations are involved in developing protocols for GMOs;

*The Codex Alimentarius Commission (Codex):* It is responsible for compiling the standards, codes of practice, guidelines and recommendations that constitute the Codex Alimentarius: the international food code. Codex is developing principles for the human health risk analysis of GM foods. The premise of these principles dictates a premarket assessment, performed on a case-by-case basis and including an evaluation of both direct effects (from the inserted gene) and unintended effects (that may arise as a consequence of insertion of the new gene). Codex principles do not have a binding effect on national legislation, but are referred to specifically in the Sanitary and Phytosanitary Agreement of the World Trade Organization (SPS Agreement), and can be used as a reference in case of trade disputes.
*The Cartagena Protocol on Biosafety (CPB):* It is an environmental treaty legally binding for its parties, regulates transboundary movements of GMOs. GM foods are within the scope of the Protocol only if they contain GMOs that are capable of transferring or replicating genetic material. The cornerstone of the CPB is a requirement that exporters seek consent from importers before the first shipment of GMOs intended for release into the environment. (http://www.who.int/foodsafety/publications/biotech/20questions/en, 2007)

1.2.2. Regulation in EU

The EU regulation on food and feed labeling is the strictest in the world. International rules for the labeling of GM foods vary considerably between nations (De Leo, 2005).

Rules concern the process of risk assessment, risk management and decision-making with regard to the release of GMOs into the environment. The new directives, Directive2001/18/EC, foresee mandatory monitoring of long-term effects associated with the interaction between GMOs and the environment. In regulations (EC) 1829/2003 and (EC) 1830/2003 the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms are regulated.

Labelling in the EU is mandatory for products derived from modern biotechnology or products containing GM organisms. Legislation also addresses the problem of accidental contamination of conventional food by GM material. It introduces a 0.9 % minimum threshold for DNA or protein resulting from genetic modification, below which labelling is not required. (http://ec.europa.eu/food/biotechnology/index_en.htm, 2007)
1.2.3. Regulation in USA

Special labelling requirements for GM foods as a class of foods are not obligated in the USA. Three U.S. agencies share responsibility for regulating agricultural biotechnology. The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) is responsible for ensuring that the growth of genetically engineered plants does not harm the agricultural environment, the Environmental Protection Agency (EPA) is responsible for assuring the human and environmental safety of pesticidal substances engineered into plants, and the Food and Drug Administration (FDA) is responsible for assuring that foods derived through genetic engineering are as safe as their traditional counterparts. (http://www.agbios.com/cstudies.php?book=REG&ev=CANUSA&chapter=USA&lang=EN, 2007)

1.2.4. Regulation in Japan

In Japan, the Ministry of Agriculture, Food and Fisheries (MAFF) and the Ministry of Health, Labor and Welfare (MHLW) administer the regulation of food safety of GMOs. Food safety assessment of GMOs is mandatory (Konig et al., 2004). A threshold of 5% for frequently used GM crops like soybean and maize was implemented (Hino, 2002).

1.2.5. Regulation in China

China signed the Cartagena Biosafety Protocol on August 8, 2000. Special labelling requirements for GM foods as a class of foods are not obligated in China. GM products must have only government approval for importation; be certified not to cause harm to humans, animals, or the environment; be for sale in the country of
origin; and contain labels noting the use of genetically modified ingredients. (Foreign Agricultural Service, 2002, http://www.agbioforum.org/v5n4/v5n4a06-curtis.htm).

1.2.6. Regulation in Turkey

Ministry of Agriculture and Rural Affairs- General Directorate of Protection and Control is the competent authority for receiving the applications for import, export of GMOs. The field trials are conducted by the Agricultural and Research Institute under the Ministry of Agriculture and Rural Affairs. (EU-TR Screening Report, 04.02.2007).

Food law (No 5179) which was focusing on controlling of all commercialised foods was implemented on 27 May 2004. Specific legislation concerning GMOs, except experimental release, has not been available. However, UN Cartagena Biosafety Protocol has been ratified by Grand National Assembly of Turkey in 2003.

Although the draft of national biosafety framework prepared by Ministry of Agriculture and Rural Affairs General Directorate of Agricultural Research (TAGEM), it is not approved by Grand National Assembly of Turkey.

1.3 Tomatoes

Tomato is an herbaceous perennial fruit with 24 diploid chromosomes, is thought to have originated in Mexico. The commercially cultivated tomato is botanically identified as *Lycopersicon esculentum* and it is a member of the *Solanaceae* family that includes potatoes, peppers, tobacco and many other plants (Jaccaud., 2003).
1.3.1. Tomato Cultivars

Wild tomato plants are still found from Ecuador to Chile, although only two have edible fruit, *Lycopersicon pimpinellifolium* (sometimes cultivated under the name of currant tomato) and *Lycopersicon esculentum* (the common tomato in wide cultivation today). There are different tomato varieties namely, *Lycopersicon esculentum* var. *cerasiforme*, *Lycopersicon esculentum* var. *pruniforme*, *Lycopersicon esculentum* var. *cerasiforme*, *Lycopersicon esculentum* var. *pyriforme*, *Lycopersicon esculentum* var. *oviforme*, *Lycopersicon esculentum* var. *ribesiformee*, *Lycopersicon esculentum* var. *vulgare*, *Lycopersicon esculentum* var. *commune*, *Lycopersicon esculentum* var. *finiens*, *Lycopersicon esculentum* var. *ummertionum*, *Lycopersicon esculentum* var. *grandifoliium* (Aybak *et al.* 2000). Small fruited type *Lycopersicon esculentum* var. *cerasiforme*, cultivated under the name of cherry tomato, is widely distributed as a wild plant in the tropics and sub-tropics (Jones, 1998).

Varieties available today for use by both the commercial such as off-season and field grown and home gardener have a wide range of plant characteristics; are resistant to many of the tomato-affected blight and wilt diseases; and are specially adapted to a particular set of growing conditions such as high tropical temperatures, field and greenhouse conditions, and fresh market versus processing tomato fruit (Jones, 1998).

Tomatoes were grown commercially in 161 countries in 2004 with a combined production of over 115 million metric tonnes. The major producers of tomatoes were China, USA, Turkey, India, Egypt, Italy and Spain. (http://www.agbios.com/dbase.php?action=ShowProd&data=5345). In Turkey, Bursa and Antalya are the major tomato producer cities. In Bursa nearly all tomatoes are procured in the fields,
whereas most of the tomatoes in Antalya are grown in the greenhouses (TURKSTAT, Agricultural department, 1999). Tomatoes are exported both as fresh and processed forms such as dried, frozen or as tomato paste, ketchup, tomato can, tomato juice and sauce from Turkey (Aybak et al. 2000).

The total number of commercially registered tomato cultivar number is 269 in 1999, in Turkey. Industrial types of tomatoes produced in Turkey are bush, indeterminate, semideterminate and cluster tomatoes. Roma-VF, H-1706, VFN8, Cal J, Ventura, Rio Grande, Chicol II, Super California, Manelle are the tomato cultivars widely grown for industrial usage. ES-58, ES-24, P378-RF, H-1370, H-1409, Earliana, H2274, SC2121, Falcon, Marmande, ACE55VF are several tomato cultivars grown in Turkey for direct consumption.

Tomato seeds have greater importance than fresh tomatoes by means of importation and exportation as the tomatoes propagate from seeds. Turkey is a major tomato producer but imports tomato seeds from different countries such as Italy, China, Israel, USA, Holland and France (Figure 1.2) according to the data taken from Turkish statistical institute TURKSTAT) (personal communication).
Figure 1.2 The countries that Turkey import tomato seeds and the amount (kg) between the years 2003 and 2005, TURKSTAT; gtip no 120991900012.

1.3.2 GM Tomatoes

Tomato is an important crop and invaluable plant model especially for genetic studies. The availability of vast genetic information and rich plant resources put the tomato in the front of attempts to evaluate it (Levin et al., 2004). By using the recombinant DNA technology, genetically modified tomatoes with variable improvements such as delayed ripening, virus resistance and insect resistance have been produced (Table 1.1). Moreover, stress tolerant tomatoes were developed to tolerate high levels of salt (Zhang et al., 2001), to resist to drought (Gupta et al., 2006) and high lycopene containing tomatoes were developed to improve the antioxidant concentration of tomato plant (Fraser et al., 2006).
Table 1.1 Approved GM Tomatoes (http://www.agbios.com/dbase.php)

<table>
<thead>
<tr>
<th>Event name</th>
<th>Company</th>
<th>Trait</th>
<th>Approved countries</th>
<th>Promoter</th>
<th>Terminator</th>
<th>NptII</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1345-4</td>
<td>DNA Plant Technology Corporation</td>
<td>Delayed ripening</td>
<td>Canada, USA, Mexico</td>
<td>35S</td>
<td>+</td>
<td>+</td>
<td>ACC synthase gene in sense orientation</td>
</tr>
<tr>
<td>351N</td>
<td>Agritope Inc.</td>
<td>Delayed ripening</td>
<td>USA</td>
<td>E8</td>
<td>+</td>
<td>+</td>
<td>Sam-k gene</td>
</tr>
<tr>
<td>5345</td>
<td>Monsanto Company</td>
<td>Resistance to lepidopteran pests.</td>
<td>Canada, USA</td>
<td>35S</td>
<td>+</td>
<td>+</td>
<td>Cry1Ac gene</td>
</tr>
<tr>
<td>8338</td>
<td>Monsanto Company</td>
<td>Delayed ripening</td>
<td>USA</td>
<td>35S</td>
<td>+</td>
<td>+</td>
<td>Acod gene</td>
</tr>
<tr>
<td>B, Da, F</td>
<td>Zeneca Seeds</td>
<td>Delayed ripening</td>
<td>Canada, Mexico, USA</td>
<td>35S</td>
<td>+</td>
<td>+</td>
<td>Antisense and sense PG gene</td>
</tr>
<tr>
<td>Flavr Savr</td>
<td>Calgene Inc.</td>
<td>Delayed ripening</td>
<td>Canada, Mexico, USA, Japan</td>
<td>35S</td>
<td>-</td>
<td>+</td>
<td>Antisense PG gene</td>
</tr>
<tr>
<td>117, 1046, 1202</td>
<td>Nivot</td>
<td>Virus resistance</td>
<td>Japan</td>
<td>No map information</td>
<td>Coat protein Cucumber Mosaic Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>405,707</td>
<td>Nivot</td>
<td>Virus resistance</td>
<td>Japan</td>
<td>No map information</td>
<td>Coat protein Cucumber Mosaic Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China tomato 1</td>
<td>Peking University</td>
<td>Virus resistance</td>
<td>China</td>
<td>No map information</td>
<td>Coat protein Cucumber Mosaic Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China tomato 2</td>
<td>CCAU</td>
<td>Delayed ripening</td>
<td>China</td>
<td>No map information</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC19, IC113</td>
<td>Zeneca</td>
<td>Delayed ripening</td>
<td>Japan</td>
<td>No map information</td>
<td>Antisense PG gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japan tomato 1</td>
<td>NIAES Planttech Research institute</td>
<td>Virus resistance</td>
<td>Japan</td>
<td>No map information</td>
<td>Coat protein Tobacco Mosaic Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No 4-7</td>
<td>Hokkaido Nat. Agr. Station</td>
<td>Virus resistance</td>
<td>Japan</td>
<td>No map information</td>
<td>Satellite RNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3.2.1 Ripening Delayed Tomato Lines

Most of the ripening delayed tomatoes are developed by targeting key enzymes of the ethylene and pectin synthesis pathways.

Ethylene is an endogenous phytohormone known to play a key role in fruit ripening in climacteric fruits, as well as many other diverse effects.

Pectin is a building block in plant cell walls and gives tomatoes their firmness. Fruit softening during ripening is due to the breakdown of cell wall pectin by an enzyme called polygalacturonase (PG).

Flavr Savr® is the first approved genetically modified tomato. It was genetically engineered to express delayed softening by insertion of an additional copy of the PG encoding gene in the “antisense” orientation, resulting in reduced translation of the endogenous PG messenger RNA (mRNA). The antisense PG gene is essentially a reverse copy of part of the native tomato PG gene that suppresses the expression of endogenous PG enzyme prior to the onset of fruit ripening. The mechanism of decreased PG activity in Flavr Savr® tomato is linked to the hybridization of antisense and sense mRNA transcripts, resulting in a decreased amount of free positive sense mRNA available for protein translation.

Transgenic tomato 1345-1 contains a truncated version of the tomato 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene, normally found in tomato. The endogenous enzyme is responsible for the conversion of s-adenosylmethionine to ACC, which is the immediate precursor of ethylene. The presence of the truncated ACC synthase gene suppresses the normal expression of the native ACC synthase gene, and while not completely understood, the mechanism
of "downregulation" is likely linked to the coordinate suppression of transcription of both the endogenous gene and the introduced truncated ACC synthase gene.

Transgenic tomato 35 1 N was developed by Agrobacterium mediated transformation in order to introduce a S-adenosylmethionine hydrolase (SAMase) encoding gene derived from E. coli bacteriophage T3 to the tomato fruit. Therefore, transformed tomatoes exhibit reduced levels of S-adenosylmethionine (SAM), the substrate for conversion (through ACC synthase) to 1-aminocyclopropane-1-carboxylic acid (ACC) which is the first committed step in ethylene biosynthesis. Lack of SAM for conversion to ACC results in tomato with reduced ethylene biosynthetic capabilities and a modified ripening phenotype.

8338 contains a gene (accd) derived from a non-pathogenic soil bacterium (Pseudomonas chlororaphis) encoding the enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd). In plants, ACCd catalyzes metabolism of 1-aminocyclopropane-1-carboxylic acid (ACC), an essential precursor for the biosynthesis of the plant hormone ethylene. The activity of ACC is sufficiently reduced in detached fruits so that ethylene becomes limiting and the ripening process is delayed.

B, Da, and F transgenic tomato lines were genetically engineered to express delayed softening by inserting a truncated version of the PG encoding gene in either the sense (lines Da and F) or the "antisense" (line B) orientation. The presence of the partial PG gene, in either sense or antisense orientation, suppresses the expression of endogenous PG enzyme at the onset of fruit ripening. The lines differ slightly in that Da and F contain the partial PG gene in the sense orientation while line B contains a partial antisense PG gene, essentially a reverse copy.
In any event, reduced PG expression decreases the breakdown of pectin and leads to fruit with slowed cell wall breakdown, better viscosity characteristics and delayed softening. Tomato lines B, Da and F have improved harvest and processing properties that allow the transgenic tomatoes to remain longer on the vine to develop their natural flavour, maintain their firmness for shipping and produce a thicker consistency in processing.

According to the information found in different web-sites, tomatoes with delayed ripening have disappeared from the market after peaking in 1998. Although it was reported that, ripening delayed tomato lines are not grown commercially in North America or in Europe anymore, in the literature and also in general, there are not any report confirming such kind of statements. (http://www.gmocompass.org/eng/groceryshopping/fruitvegetables/15.genetically_modified_tomatoes.html, http://www.gmo-watch.com/pages/tomatoes.asp)

1.3.2.2 Virus Resistant Tomato Lines

According to the data in the BATS reports, virus resistant tomatoes were developed and authorized in China and Japan. Genetic map information and also the transformation methods used to develop these tomato lines are not available.

Japan tomato 1 contains coat protein coding gene of tobacco mosaic virus. Commercial seed and field production of this line is legal, but there is not any authorization for marketing (Food approval is needed).

Coat protein coding genes of cucumber mosaic virus is inserted to 405 and 707 lines. They are not authorized for marketing as food.
China tomato 1 was developed by inserting cucumber mosaic virus coat protein coding gene. It has been approved for marketing in China (BATS, 30/06/2003).

1.3.2.3 Insect Resistant Tomato Line and the Role of *Bacillus thuringiensis*

Tomato is extensively damaged by the lepidopteran insect *Helicoverpa armigera Hubner*, also called tomato fruit borer. The adults lay eggs on the leaves and lower buds. The larvae upon hatching bore into the developing fruits causing severe damage and thereby reducing the marketable yield. (Chakrabarti *et al.*, 1998).

*Bacillus thuringiensis* (Bt), a Gram-positive, spore-forming bacterium, produces a variety of insecticidal crystal proteins (ICP) toxic to lepidopteran insects (Kumar *et al.*, 1996). These crystalline proteins are highly insecticidal at very low concentrations (Schnepf, 1998). As these proteins are non-toxic to mammals and other organisms so far, Bt strains and their insecticidal crystal proteins (ICPs) have acquired acceptability as eco-friendly biopesticides all over the world and have been under extensive use in agriculture for the past four decades (Schnepf, 1995). The delivery of Bt ICPs through spray formulations, engineered Bt and other bacteria has certain limitations. The biopesticidal sprays have some disadvantages such as short half-life, physical removal by wind and rain and inability to reach burrowing insects. With the advent of molecular biology and genetic engineering, it became possible to use Bt more effectively by introducing the ICPs of Bt in crop plants.

Expression of Bt genes in tomato was one of the first examples of genetically modified plants with resistance to insects (Fischhoff *et al.*, 1987). Tomato plants expressing Cry1A(b) and Cry1A(c) genes are effective against the lepidopteran insects (Delannay *et al.*, 1989; Van der Salm *et al.*, 1994).
However, the level of toxin expression in plants was insufficient when the native Cry genes were used. A significant breakthrough was made in 1990 by researchers at Monsanto Company (USA) who modified the cry1Ac gene for better expression in plant cells (Perlak, 1991). The codon usage of prokaryotic genes of Bt was altered to resemble that of higher plants. Bt genes are adenine-thymine rich while plant genes tend to have a higher guanine-cytosine content. The expression of insecticidal proteins has been enhanced by increasing guanine-cytosine content of their encoding genes without changing the amino acid sequence (Sharma et al., 2000). The use of a truncated version of the genes and modification of the coding sequence, such as removal of potential RNA processing and polyadenylation signals and optimization of the codon usage let high levels of toxin expression in plants (Perlak et al., 1990; Koziel et al., 1993; Fujimoto et al., 1993; Jansens et al., 1995; Kumar et al., 1998). In short, more convincing results were obtained with the synthetic genes (Jouanin et al., 1998).

Insect-resistant authorized tomato line 5345 was developed to express the insecticidal protein, Cry1Ac, encoded by the cry1Ac gene from the soil bacterium Bacillus thuringiensis subsp. kurstaki HD73. Delta-endotoxins, such as the Cry1Ac protein expressed in tomato line 5345, exhibit high insecticidal activity.
Figure 1.3 Genetic construct of insect resistant tomato line 5345 (Bruderer & Leitner, 2003)

1.4 Detection Methods of GM Tomatoes

As more GM foods, food ingredients, and additives are introduced to the markets, nearly 40 countries and areas have issued GMO labelling regulations.

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

The development and application of reliable detection and quantitative analytical methods is essential for the implementation of labelling rules especially in countries where GM labelling is mandatory.
Some of important steps in GMO detection process are sampling and sample preparation (Anklam et al., 2002). The sampling procedure determines how representative the results will be, whereas quality and quantity of analytes may vary depending on the sample preparation.

Several methods have been developed either based on DNA detection, or on protein detection. Detection methods vary in their reliability, robustness and reproducibility; in combination with different levels of cost, complexity, and speed, and there is no one method that is applicable to all circumstances (Anklam et al., 2002). However, PCR based methods are the most preferred method used for GM detection according to the studies found in the literature and the reports.

To execute GMO labelling policies successfully, qualitative and quantitative PCR methods are well established for GMO detection through screening the universal elements, or detecting target genes and event-specific DNA fragments of the inserted heterologous DNAs in GM maize lines (Bt11, Event176, Mon810, T25 and GA21) and GMsoybean (Zimmermann et al., 1998; Hernandez et al., 2003). However, up to now there are few reports about the screening or specific PCR detection methods for GM tomato (Hemmer et al., 1997; Meyer et al., 1995).

The Flavr Savr tomato contains a resistance gene for the antibiotic kanamycin (kansur) and a gene construct for the inhibition of fruit ripening and softening (antisense polygalacturonase). A method was developed by Meyer (1995) in order to detect the presence of transgenic elements found in Flavr Savr tomatoes.

Also specialised reports on detection methods for genetic elements used in the generation of transgenic tomato are developed by Pietsch and Waiblinger in 1996. Specific primer sets to detect ripening delayed GM tomatoes can be found in the

GM tomato Huafan No 1 with a character of long shelf-life was the first GM plant which was approved for commercialization in China, in 1996. Screening and construct-specific PCR detection methods for detecting the universal elements transformed into tomato, such as cauliflower mosaic virus 35S (CaMV35s) promoter and the nopaline synthase (NOS) terminator of Agrobacterium tumefaciens, and the specifically inserted heterologous DNA sequence between CaMV35s promoter and anti-sense ethylene-forming enzyme (EFE) gene were set up. They also found a single copy endogenous reference gene for tomato, LAT52, in order to use in Real-Time PCR detection systems.

1.4.1 DNA Based Methods

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

PCR allows the million-fold amplification of a target DNA fragment in a highly sensitive and specific manner. Confirmation of the identity of a PCR amplicon is necessary in order to ensure that the amplified DNA product corresponds to the chosen target sequence and is not a product of non-specific binding of the primers. The simplest approach is to control whether the PCR products have the expected size is by gel electrophoresis. However, there is a risk that an artifact of the same size as the target sequence has been amplified. Several methods are available for this purpose such as restriction enzyme digestion, sequence analysis, southern blot assay, nested PCR etc (Anklam et al., 2002).
Any PCR-based detection strategy depends on a detailed knowledge of the transgenic DNA sequences and of the molecular structure of the GMOs in order to select the appropriate oligonucleotide primers. For routine screening purposes, genetic control elements such as the Cauliflower mosaic virus (CaMV) 35S promoter (P-35S) and Agrobacterium tumefaciens NOS terminator and neomycin phosphotransferase (nptII) selection marker are used. (Bruderer et al., 2003)

To adapt these GMO labeling requirements, four general types of PCR detection strategies, i.e., screening-, gene-, construct-, and event-specific PCR detection methods (Figure 1.4), have been developed to discriminate between GM- and non-GM-derived DNA varieties (Vaughn et al., 2005).

![Figure 1.4 Scheme of qualitative PCR systems. Four types of PCR systems showing increased specificity from top to bottom. (H, host genome; P, promoter; E, enhancer; G, gene of interest, T, terminator) (Holst-Jensen et al., 2002).](image-url)
1.4.1.1 Screening PCR

Screening of GMOs in order to gain a first insight into the composition of the food as well as the agricultural product. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results. For this purposes, commonly used regulatory elements such as 35S promoter, NOS terminator and marker genes such as antibiotic resistance genes are screened as the first step of detection. Targeting the P-35S, T-NOS or NPTII have wide application for screening genetically modified organisms. However, these results can not be used to identify the GMO, since presence of one of the screening targets does not necessarily imply the presence of GMO-derived DNA. The source of P-35S or T-NOS could be naturally occurring CaMV and it is generally believed that Agrobacterium or other soil bacteria containing one or more of the targets are present in the soil.

1.4.1.2 Gene Specific PCR

PCR methods targeting the gene of interest are more specific than screening PCR systems. Inserted gene that gives a proper characteristic to the donor organisms is the target of gene specific PCR system.

1.4.1.3 Construct Specific PCR

Target of these construct specific PCR systems are junctions between adjacent elements of the gene construct, like the promoter and the gene of interest. Four construct specific PCR systems that specifically detect Bt11, Event176, Mon810 and Liberty maize lines were developed by Yamaguchi et al., 2003. For each maize line,
specific primer sets that cover the junctions between elements are used and the results of construct specific PCRs were compared with ELISA technique results.

Gene- and construct-specific methods are more specific but may cause false positives when the modified genes are used in other GMOs with variable copy number, and these methods cannot distinguish between different GMOs if the same construct has been integrated (Ronning et al., 2003).

1.4.1.4 Event Specific PCR

The only unique signature of a transformation event, within the limitations of present day technology, is the junction at the integration locus between the recipient genome and the inserted DNA. That is why event specific PCR is performed to overcome these problems. Until now, several event-specific quantitative PCR methods have been established for GM crops, such as, Roundup Ready soybean (Berdal et al., 2001; Taverniers et al., 2001; Harris et al., 2001), MON810 (Hernandez et al., 2003; Holck et al., 2002), Bt11 (Ronning et al., 2003), Starlink (Windels et al., 2003), NK603 maize (Huang et al., 2004), Mon1445 and Mon531 cotton (Yang et al., 2005), T45 canola (Yang et al., 2006), Huafan no:1 (Litao et al., 2005).

Multiplex-PCR technique is another approach used for GMO detection. By using this method it is possible to amplify different target genes in one PCR reaction. Detection of nine soybean, six maize, seven potato and two rice samples were done by multiplex-PCR. After the amplification of samples with biotinylated screening and identification primers, the multiplex PCR products and membrane bound oligonucleotide probes were hybridized. This multiplex polymerase chain reaction/membrane hybridization assay improves detection efficiency obviously,
reduces false negative or false positive results, and improves the result accuracy of GMOs detection effectively (Su et al., 2003).

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

Alternative techniques for GMO analysis include: Chromatography, Near infrared spectroscopy and DNA chips.

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

To cope up with rapid development of GM plants, new technologies and instruments will be needed for the high throughput and low cost detection of an increasing variety of genes (Anklam et al., 2000).

Real time PCR is the most commonly used technology for quantification of GM crop content. The amount of product synthesized during the PCR is measured in real time by detection of the fluorescence signal produced as a result of the amplification. It has been shown empirically that the concentration of DNA in RT-
PCR reaction is proportional to PCR cycle number during the exponential phase of PCR (De Leo et al., 2005).

1.4.2 Protein Based Detection Methods

Detection of a novel protein synthesized by a gene introduced during transformation constitutes an alternative approach for the identification of genetically modified plants. Protein detection methods are based mainly on immunoassays (antigen and antibody) and since they require proteins with an intact tertiary or quaternary structure, these methods are limited to fresh and unprocessed foods (Bonfini et al., 2001).

The crucial component of an immunoassay is an antibody with high specificity for the target protein (antigen). Immunoassays can be highly specific and samples often need only a simple preparation before being analysed. Moreover, immunoassays can be used qualitatively or quantitatively over a wide range of concentrations. However, protein based methods are unable to detect a genetic modification if the modified gene is inactive in the cells from which an analytical sample is derived (Miraglia et al., 2004). Another problem is the processing of the food, as the antibodies only recognize three dimensional protein structures (Pooping et al., 2006).

The most common type of immunoassay is the ELISA, which utilizes an enzyme-labeled immunoreactant (antigen or antibody) and an immunosorbent (antigen or antibody bound to a solid support). It offers high degree of automation and a high throughput of samples. (Bonfini et al, 2001).
Lateral flow strips is an easy way to detect GMO. This technology offers a semi-quantitative detection of GMO even in the field as an initial screening method (Farid, 2002). However, commercially available lateral flow strips are currently limited to few GM products.

Western Blotting is also highly specific method providing qualitative results suitable to determine target protein. However, it is not suitable for routine testing of GMOs. (Farid, 2002).

1.5. Aim of the Study

Turkey is one the fourth biggest tomato producer country in the world and Turkey import tomato seeds mostly from Italy, China, Israel, USA, Holland and France.

The aim of this study is to analyze and evaluate tomato samples collected arbitrarily from different parts of Turkish food markets and bazaars. Firstly, DNAs of the collected 40 fresh tomato samples and 35 seed samples were isolated and then screened for 35S promoter, NOS terminator and NPTII marker genes by using PCR methods with eight different primer sets. Real time PCR is used to confirm 35S and NOS positive results obtained from conventional PCR.

Secondly, samples were analyzed to identify if they are modified with sam-k, F type truncated PG or cry1Ac genes. F type truncated PG specific primers were used by Meyer (1999) and sam-k specific primers were used in our laboratory previously by Türkoglu (2007). Although there area a lot of studies on Bt crops detection in the literature, a study on detection of 5345-1 GM tomato line, carrying cry1Ac gene, could not found. Primers for the identification of cry1Ac gene were designed and PCR analyses were carried out. Then, construct specific PCR primers for 35 1 N and
5345-1 lines were designed for further verification. Results were sent to sequence analysis for confirmation.
CHAPTER II

MATERIALS & METHODS

2.1. Tomato Samples

Both fresh tomato samples and tomato seeds were obtained randomly from different markets both bazaar and supermarkets in different regions of Turkey. Samples were obtained between the years 2005 and 2007. Additionally some tomato samples were obtained from USA, Iran and Greece for comparative study. A total of 40 fresh tomato samples and 35 seed samples were collected.

Figure 2.1 Some of the collected fresh tomato samples.
2.2. Reference Materials

As positive controls, genetically modified tomato seeds which contain 35S promoter, Nos terminator and NptII resistance gene were kindly obtained from Dr. Chris Bowler, Laboratory of Molecular Plant Biology Stazione Zoologica- Italy and from Max-Planck Research Institute, Germany. Plasmids1320 containing 35s and NOS regions, p2300 containing NPTII region designed by the company Cambia (Canberra, Australia) also obtained from Genetic Laboratory in METU Food Engineering Department. Plasmid ROKII is obtained from Plant Genetics Laboratory in METU Biology Department. As negative control, tomato seeds obtained from Cappadocia Region were used.

In order to use cry1Ac gene as a positive control, Bacillus thuringiensis subsp. kurstaki HD73 and E.coli clone carrying cry1Ac region (BGSC No: 4D4, ECE53)
were kindly provided by *Bacillus* Genetic Stock Center, The Ohio State University, USA

CTAB DNA isolation protocols both from fresh tissue and from dry tissue were used for DNA extraction. DNA extraction kits are also used in order to isolate pure DNA from fresh tomato samples.

### 2.2.1. DNA Isolation from Fresh Tomato Samples

DNA isolation from fresh tomato tissues were done according to the modified CTAB extraction procedure of Doyle & Doyle (1990) which provides easy and rapid DNA extraction. Each tomato was washed in running water to remove adhering soil particles. They were cut into 2 halves with sterile knife, seeds and the inner fruit parts except the outer skin layer were used for DNA isolation. They were ground in sterile mortal and pestle using liquid nitrogen to get a homogenous powder. 5-7.5 ml CTAB isolation buffer (2% (w/v) CTAB (Applichem), 1.4 M NaCl (Merck), 20 mM EDTA (Sigma), 100 mM Tris-HCl (Sigma) pH:8.0 and 1% (w/v) PVP-40 (Sigma)) with freshly added 1% (v/v) β-mercaptoethanol (Applichem) was preheated in a 50 ml falcon tube to 60 °C in a water bath. 2.5-5 g homogenized sample and the preheated buffer were mixed and placed at 60°C for 2 hours. After incubation samples were extracted with an equal volume of chloroform-isoamyl alcohol (Applichem, (24:1), (v:v)), mixed gently and centrifuged at Sorvall centrifuge at 10000 rpm for 5 min. The aqueous phase was removed with a pipet to a new eppendorf tube and then, this chloroform-isoamyl alcohol extraction step repeated 3 times. 2/3 volumes of cold isopropanol was added to the clear supernatant and mixed gently to precipitate the nucleic acids. After centrifugation at 10000 rpm for 2 min, the supernatant was discarded and 10-20 ml washing buffer (75% v/v ethanol (Delta Kimya), 10mM ammonium acetate (Applichem)) was added. The pellet was washed for minimum 20 minutes and centrifuged at 6000 rpm for 5 minutes. The
supernatant was poured off carefully and the pellet was allowed to air dry at room temperature. The dried pellet was resuspended in double distilled sterile water.

Several steps from the procedure developed by Doyle & Doyle in this DNA isolation procedure were modified. The amount of the sample homogenized was increased and the duration of incubation is increased. Also the chloroform-isoamylalcohol extraction step is repeated three times in order to obtain more pure and higher amount of DNA.

DNA of fresh tomato samples were also extracted by using CONGEN Surefood DNA extraction kit by following the procedure provided by the manufacturers.

2.2.2 DNA Isolation from Dry Tissue

First of all, in order to prevent potential bacterial contamination originating from the soil, surface sterilization is performed for seed samples. According to the study carried out by Koç, et al., (2007) seeds were washed for 1 min with 70% ethanol then soaked for 15 min in 5% sodium hypochloride solution followed by four washes of sterile distilled water before extracting DNA from seeds.

In this study, DNA extraction from tomato seed samples was performed by CTAB DNA isolation procedure (Querci et al., 2004). 200 mg of seeds were ground in sterile mortar and pestle using liquid nitrogen to get a homogenous powder and transferred into a sterile 2 ml microcentrifuge tube, 300 ul of sterile deionized water and 500 ul CTAB-buffer pH 8.0 (20 g/l CTAB (Applichem), 1.4 M NaCl (Merck), 0.1 M Tris-HCl (Sigma), 20 mM Na2EDTA (Sigma)) were added to microcentrifuge tube and mixed with a loop after each addition. 20 ul Proteinase K (20 mg/ml, MBI Fermentas) was added, mixed and placed at 65°C overnight. After incubation 20 ul
RNase A (10 mg/ml, MBI Fermentas) was added, mixed and again kept at 65°C for
5-10 min. After incubation, samples were centrifuged (Hettich Zentrifugen Mikro
12-24) for 10 min at about 16,000 x g and the supernatant was transferred to a
microcentrifuge tube containing 500 µl chloroform-isoamylalcohol (24:1) v/v,
gently mixed for 30s and again centrifuged for 10 min at about 16,000xg. 500 µl of
upper layer was transferred into a new microcentrifuge tube and this extraction step
is repeated three times. The upper layer was transferred to a microcentrifuge tube
and 2 volumes of CTAB precipitation solution pH 8.0 (5 g/l CTAB (Applichem),
0.04 M NaCl (Merek)) was added, mixed by pipetting. After incubation for 60 min
at room temperature, samples were centrifuged for 5 min at 16,000 x g, the
supernatant was discarded and the precipitate was dissolved in 350 µl NaCl (1.2 M,
(Merek)). 350 µl chloroform (Applichem) was added and mixed for 30s, centrifuged
for 10 min at 16,000xg until phase separation occurs, the upper layer was transferred
to a new microcentrifuge tube, 0.6 volumes of isopropanol (Delta Kimya) was added
and mixed. After a centrifugation for 10 min at 16,000 x g, the supernatant was
discarded. 500 µl of 70% ethanol (Delta Kimya) solution was added and mixed
carefully, centrifuged for 10 min at 16,000 x g. The supernatant was again
discarded, the pellet was dried and DNA was re-dissolved in 100 µl sterile deionized
water.

2.2.3 Plasmid DNA isolation

- Transformation of competent cells

First of all in order to multiply the plasmid DNA, plasmid was transformed to the
E.coli. 0.5ul plasmid was mixed with 50ul TE buffer (pH:8). 300ul competent cells
were mixed with plasmid and TE buffer solution and kept on ice for 30 minutes. The
mix was transferred into 42°C water bath for precisely 90 seconds. 1ml LB broth was
added and kept at 37°C for 1 hour. 50-200 µl aliquots were finally spread on kanamycin containing LB plates (1:100) and overnight incubated at 37°C.

- Alkaline lysis extraction

After transformation of competent cells, alkaline lysis extraction procedure was followed to obtain plasmid DNA.

5 ml LB broth and 2.5 µl kanamycin was added to the falcon tube and inoculated with a colony before overnight incubation at 37°C. In order to burst cells and to separate from broth, they were centrifuged at 6000 rpm for 8 minutes. Cells were resuspended at 200 µl Solution I (AppendixB) then incubated at room temperature for 15 minutes. After 200 µl Solution II (AppendixB) added, gently mixed for 7-8 times in order to destroy genomic DNA of bacteria but not the plasmid DNA. After 15 seconds, it was incubated on ice absolutely 5 minutes. 200 µl Solution III (AppendixB) was added and mixed gently for 7-8 times and incubated 15 minutes on ice. Then, centrifuged at 13000 rpm for 10 minutes at 4°C. Supernatant was transformed into new eppendorf tube (400 µl/eppendorf). 2 volume of cold ethanol was added into eppendorf tubes and incubated 1h at -20°C. As a last step, tubes were centrifuged 10 minutes at 13000 rpm, and supernatant was discarded. After pellet was air-dried for 5 minutes, dissolved on 15 µl sterile distilled water. Plasmid DNAs were used in the optimization of PCR conditions since the positive control tomato DNA was limited in quantity.

2.3. Determining the DNA Purity

The purity and the amount of isolated DNA were determined by measuring its absorbance by using an U.V spectrophotometer.
After blank was measured by sterile dH₂O and calibrated as zero, DNA was diluted with sterile dH₂O in the ratio of 1/200 and placed in a cuvette. Absorbance was measured at 260 nm and 280 nm. The ratio of A₂₆₀/A₂₈₀ represents the purity of the sample (ratios of 1.8=pure, 2.0=RNA contamination, 1.6≤ protein contamination). DNA concentrations were expressed as ng/μl. Formula used for determination of DNA concentration was as below. OD₂₆₀ stands for optical density in A₂₆₀ and 50 represents the A₂₆₀ unit of ds DNA (Somma, 2002).

\[
\text{DNA Concentration} = \text{OD}_{260} \times 50 \times \text{Dilution factor}
\]

Beside DNA purity measurements by using an UV spectrophotometer, isolated DNAs were run on 0.8% agarose gels to check the resolution, molecular weight and whether they were intact.

2.4. Primer Design

PatF/PatR primer set, producing 124 bp fragment, has been previously designed by Jaccaud et al., (2003) was used to detect Patatin gene.

Three different 35S primer sets were used for the detection of 35S Promoter region. P35S-afu/P35S-ar1 primer set produces an amplicon of 207 bp in length (Bonfini., 2007) and 35S-F/35S-R primer set produces 195 bp amplicon (European Commission, JRC). Also 35SEU-F/ 35SEU-R primer set was designed in to screen 35S promoter region by using primer3 primer design programme. This primer set produces a 391 bp amplicon.

Two different primer sets were used for screening NOS terminator region. Nos-F/ Nos-R primer set produces a 180 bp amplicon in length (European Commission,
JRC) and newly designed primer set Nos207-f/ Nos207-r produces a 207 bp fragment.

Three different primer sets producing 411 bp and 459 bp amplicons were used for NPTII detection. Kn-F/ Kn-R primer set producing 459 bp fragment designed by Sonmezalp, (2004). KAN411F/ KAN41R primer set producing 411 bp amplicon was used in a previous study of CORESTA for tobacco and TN5fw/TN5rv primer set producing 175 bp amplicon was validated by Federal Institute for Health Protection of Consumers (BgVV).

Primers used to detect 35S promoter, NOS terminator, cry1Ac-pE35S promoter junction region and the Cry1Ac genes were designed by using the software programmes ClustalW (ClustalW homology search programme, and Primer3 (Primer3 primer design programme). ClustalW programme was used to check homologous regions of sequences found from NCBI gene bank and the Primer3 programme was used to design specific primers that could match to these homologous regions.

The screening reaction for ripening delayed tomatoes was performed with primer pair PG34L/PG34R amplifying part of the F type PG gene (non-transgenics give 380 bp whereas transgenics give 180+380 bp amplicons) (Bonfini., 2007).

Sam3f/ Sam5f primer set producing a 217 bp amplicon was used for screening sam-k gene. This primer sets was previously used in our laboratory by Türkoğlu (2007) according to the advice of Efendi (Bogor Agricultural University, Bogor, Indonesia).

The primers to identify cry1Ac gene was designed by using the primer3 primer design programme after the sequence of natural and synthetic cry1Ac found in
NCBI Gene Bank. The cry1Ac gene transformed in transgenic tomato lines is synthetic and its homology with natural cry1Ac gene from *Bacillus thuringiensis* subsp. *kurstaki* HD73 was compared. Primer set was designed between the homologous region of natural and synthetic cry1Ac genes in order to amplify both positive control DNA and the expected tomato samples (Appendix M).

Construct specific Crycs primer set was also designed for verifying the construct region in between cry1Ac and PE-35S promoter region and samcs primer set was designed for the verification of 35 1 N line after the identification PCR studies.

All primer sets used in this study were presented in Table 2.1 and 2.2. Primers were synthesized by Iontek (Istanbul, Turkey) and GENSUTEK (Ankara) and stored at -20°C.
<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Target size (bp)</th>
<th>Accession No</th>
<th>Reported by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patatin</td>
<td>Pat-F</td>
<td>ccataggccatggcactgtgtaagacttgcacagtgc</td>
<td>124</td>
<td>X03932</td>
<td>Jaccaud et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Pat-R</td>
<td>gtaagacttgcacagtgcctggttc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35S-P</td>
<td>35S-F</td>
<td>gctctcaaatgtggtactgtgca</td>
<td>195</td>
<td>AF078810</td>
<td>European Commission (JRC)</td>
</tr>
<tr>
<td></td>
<td>p35s-af1u</td>
<td>ccataaatgtgcctgtgtctgtgca</td>
<td>207</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>p35s-ar1</td>
<td>ggtgtggattggtggtgca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOS-T</td>
<td>35SEU-F</td>
<td>gattgttgtgataaatgtgtgca</td>
<td>391</td>
<td>U12540</td>
<td>European Commission (JRC)</td>
</tr>
<tr>
<td></td>
<td>35SEU-R</td>
<td>ggattgtgtgataaatgtgtgca</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Nos-F</td>
<td>gatccctgtgtgctgtgtgtgactgtgca</td>
<td>180</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nos-R</td>
<td>ttatctgctgtctgtgactgtgca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nos207-f</td>
<td>tgcctcaaacatgtgtgcaat</td>
<td>207</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Nos207-r</td>
<td>ttccctgctgtctgtgactgtgca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPTII</td>
<td>Kn-F</td>
<td>ttgctctgtgctgtgtgtgca</td>
<td>459</td>
<td>AF274974</td>
<td>Sonmezalp (2004)</td>
</tr>
<tr>
<td></td>
<td>Kn-R</td>
<td>gacacacctgtgctgtgtgtgca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>KAN411F</td>
<td>gctggcactggtgactgtgtgtgca</td>
<td>411</td>
<td></td>
<td>CORESTA</td>
</tr>
<tr>
<td></td>
<td>KAN41R</td>
<td>ggcggcactggtgactgtgtgtgca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TN5fw</td>
<td>gcatactggctgtctgctgactttt</td>
<td>175</td>
<td></td>
<td>Federal Institute for Health Protection of Consumers (BvYV)</td>
</tr>
<tr>
<td></td>
<td>TN5rv</td>
<td>gcatactggctgtctgctgactttt</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Primers used for identification studies

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Target size (bp)</th>
<th>Accession No</th>
<th>Reported by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samk</td>
<td>Sam5f,</td>
<td>gacctccgagatgaaccga accggctgattcagtgacccc</td>
<td>217</td>
<td>X04791</td>
<td>Bogor Agri. Univ. Indonesia 2007</td>
</tr>
<tr>
<td></td>
<td>Sam3f</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>Pg34f,</td>
<td>gatcctgagctgcatctagt gcctgcccatcctgcatgg</td>
<td>380+180</td>
<td>X05656</td>
<td>Bonfini L., 2007</td>
</tr>
<tr>
<td></td>
<td>Pg34r</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry1ac</td>
<td>Cry323f,</td>
<td>ggcctgtagagacaggtcatca agtgtgctaggg</td>
<td>329</td>
<td>U63372</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Cry1323r</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cryes</td>
<td>Cryesf,</td>
<td>gcgagaatgtcgcaaggaag gtggtgcaagaggtgaa</td>
<td>713</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Cryesr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sames</td>
<td>sameuf,</td>
<td>gtagaatgtagatgtgaggcgttcctggctctggtaatgg</td>
<td>400</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>sameucr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sameuf,</td>
<td>tgctacggtgcttccgcctctc           tcgcgcgcatatattttt</td>
<td>600</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>sameucr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5. Polymerase Chain Reaction (PCR)

Two – five microliters of the isolated tomato DNA were added to the reaction mixture and a final volume of 30μl was used in all PCR assays. The reaction mixture contained double distilled water, 1X PCR buffer, MgCl₂, dNTP mix (Fermentas # R0191), primer pairs and Taq DNA polymerase (Fermentas # EP0405).

For each PCR set, a negative control was always included to check reagent contamination. In the negative control tube, no template DNA was added, instead
double distilled water was added to fill up 30µl reaction volume. Heating was done in 0.2 ml thin wall PCR tubes (Axygen, USA), using Thermal cycler TC-412 (TECHNE, USA) and Biorad MJ Mini.

2.6. Amplification of Target Genes by Polymerase Chain Reaction

All amplification reactions were performed in 30µl volume, using Techne and Biorad thermocyclers. The DNA amount used in PCR amplifications were 5 ng for control PCR, 20 ng for screening and 100 ng for identification PCRs.

In order to obtain sensitive and specific detection, cycling conditions like annealing temperatures, denaturation, annealing and extension times, cycle numbers were checked. PCR parameters including MgCl₂ and primer concentrations were optimized by performing the same PCR with variable concentrations of each parameters using gradient program of Biorad MJ Mini. PCR cycling conditions mentioned in below tables 2.3, 2.4, 2.5, 2.6, 2.7 are all optimized parameters.

2.6.1. Control PCR

In order to avoid false negatives, Patatin specific PCR was conducted to determine if isolated tomato DNA was amplifiable or not. Primer sets Pat- F/Pat- R were used to target patatin protein, a major storage protein in tomatoes. Primer set specific to patatin gene has been previously designed by Jaccaud et al. (2003) against the sequence NCBI Gene bank accession no X03932 (Table 2.1).

PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 1.5mM MgCl₂ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 20 pmol Patatin forward primer, 20
pmol Patatin reverse primer, 1 unit \textit{Taq DNA Polymerase} enzyme, and ddH$_2$O up to 30 ul. Cycling conditions were mentioned in Table 2.3 for patatin control PCR.

Table 2.3 PCR conditions for patatin

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>53°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles:</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

2.6.2. Screening PCRs

Screening of genetically modified tomatoes target three genes; 35S promoter, NOS terminator and NPTII kanamycin resistance gene and 8 different primer pairs were used for this purpose.

2.6.2.1. 35S Promoter Specific PCR

Three different 35S promoter specific primer sets were used to screen.

PCR reaction mix components with following final concentrations were collected in a 200 ul sterile PCR tube for both 35S promotor primer sets: 1X PCR Buffer (MBI Fermentas), 1.5mM MgCl$_2$ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 60 pmol 35S forward primer, 60 pmol 35S reverse primer, 1 unit \textit{Taq DNA Polymerase}
enzyme, and ddH₂O up to 30 µl. Cycling conditions were mentioned in Table 2.4 and 2.5 for 35S promotor screening PCRs.

Table 2.4 PCR conditions for P-35S using 35SF1/R2 and p35S-af1u/p35S-ar1 primer sets

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>54°C</td>
<td>55 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles:</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2.5 PCR conditions for 35S- Promoter using 35S EU F/R primer set

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles:</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>
2.6.2.2. NOS Terminator Specific PCR

Primer sets NOS-F/NOS-R and NOS207F/R was used for detection and amplification of NOS terminator gene.

PCR reaction mix components with following final concentrations were collected in a 200 μl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 3 mM MgCl₂ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 60 pmol NOS forward primer, 60 pmol NOS reverse primer, 1 unit Taq DNA Polymerase enzyme, and ddH₂O up to 30 μl. Same ratios are used for both NOS terminator primer sets.

Cycling conditions were mentioned in Table 2.6 and 2.7 for NOS terminator screening PCR.

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>56°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

Number of cycles: 40
Table 2.7 PCR conditions for NOS terminator using NOS207 f/NOS207r primer set.

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>1 min.</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles: 45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6.2.3. NptII Specific PCR

Detection of nptII gene was studied using primer sets Kn-F and Kn- R, KAN411F, KAN411R and TN5fw, TN5rv. The concentrations of reaction components were same for both three primer sets.

PCR reaction mix components with following final concentrations were collected in a 200 µl sterile PCR tube: 1X PCR Buffer (MBI Fermentas), 3 mM MgCl₂ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 60 pmol NOS forward primer, 60 pmol NOS reverse primer, 1 unit *Taq DNA Polymerase* enzyme, and ddH₂O up to 30 µl.

Thermal cycling conditions for Kn-F/R, KAN411F/R and TN5fw/rv are shown in Table 2.8 and 2.9.
Table 2.8 PCR conditions for NPTII gene region using Kn-F/R, KAN411F/R primer sets

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>54°C</td>
<td>55 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles:</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2.9 PCR conditions for NPTII gene region using TN5-fw/rv primer sets

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles:</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

2.6.3 Identification PCRs (Gene specific target)

Three different primer sets were used to identify PG, sam-K, cry1Ac genes respectively.
The first primer set was used in order to identify truncated polygalacturonase gene which found in F type ripening delayed GM tomatoes. The control and screening reaction was performed with the primer pair PG34L/PG34 R, which amplifies part of the PG-gene. Non-transgenic tomato samples produces 383 base pair PCR product whereas modified ones produces 180 base pair.

The second primer set was used to identify 35 S N transgenic line. For this purpose sam-k protein coding gene specific primers were used. This primer set produces 217 bp PCR products.

The last identification primer set was designed for cry1Ac gene which is found in authorized 5345-1 Bt tomatoes. In order to design primers NCBI Gene Bank was used to find related sequences and the CLUSTALW multiple sequence alignment programme was used to compare the homologous sequences of synthetic cry1Ac and the natural cry1Ac genes. Designed primers were amplifying for both natural and the synthetic cry1Ac genes. The expected amplicon length was 323 bp for Cry1Ac sequences.

PCR reaction mix components with following final concentrations were collected in 200 μl sterile PCR tube for all of the identification PCRs: 1X PCR Buffer (MBI Fermentas), 3mM MgCl₂ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 60 pmol forward primer, 60 pmol reverse primer, 1 unit Taq DNA Polymerase enzyme, 100 ng DNA and ddH₂O up to 30 μl for each reaction.

Cycling conditions for identification PCRs were illustrated in Table 2.10 and 2.11.
Table 2.10 PCR conditions for PG gene region using PG34L/PG34 R primer set.

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>10 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>1 min.</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min.</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>6 min</td>
</tr>
<tr>
<td>Number of cycles: 40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.11 PCR conditions for sam-k and cry1Ac gene region using sam3f/5f and cry323f/r primer sets respectively.

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>56°C</td>
<td>50 sec.</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 sec.</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles: 45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.6.4. Construct-specific PCR

2.6.4.1 Construct Specific PCR System for 5345-1 line

In order to confirm the results obtained from the screening and identification studies, construct specific PCRs were carried out. For this purpose cry1Ac gene carrying samples were used and the 5345-1 line was targeted. According the genetic map of 5345-1 line, cry1Ac is near the pE-35S promoter (Figure1.2). Construct specific primer sets were designed for the junction region in between the cry1Ac gene and the pE-35S promoter. The expected amplification product was nearly 700 bp.

PCR reaction mix components with following final concentrations were collected in 200 μl sterile PCR tube for construct specific PCR: 1X PCR Buffer (MBI Fermentas), 1.5mM MgCl₂ (MBI Fermentas), 0.2mM dNTP (MBI Fermentas), 30 pmol forward primer, 30 pmol reverse primer, 1 unit Taq DNA Polymerase enzyme, 100 ng DNA and ddH₂O up to 30 μl for each reaction.

2.6.4.2 Construct Specific PCR System for 351 N line

In order to confirm the results obtained from the screening and identification results, construct specific PCR was carried out. For this purpose sam-k gene carrying seed sample SE17 was used and the 351 N line was targeted. According the genetic map of 351 N line sam-k gene is near the NOS terminator region (Figure1.5). Construct specific primer sets were designed for the junction region in between the sam-k gene and the NOS terminator. The expected amplification product is nearly 400 bp.
Figure 2.3 Genetic construct of ripening delayed tomato line 35 1 N (Bruderer & Leitner, 2003)

Cycling condition for construct specific PCRs was illustrated in Table 2.12.

Table 2.12 Construct specific PCR conditions using escryf/r and sameuf/r primer sets

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>Number of cycles: 40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR products obtained from the construct specific PCR studies were containing extra bands. Before sequence analysis in order to confirm the construct, unrelated
bands should be removed from the expected bands. Removal of unspecific bands from the expected bands obtained from construct specific PCR products was achieved by using Fermentas gel extraction kit. Kit procedure was followed to extract the related bands.

2.7. Visualization on Agarose Gels

PCR products were analyzed on 2% agarose (Prona Basica Le) gels prepared and run in 1X TAE. 100 bp DNA Ladder (Fermentas) was used as DNA marker. All gels were run at 80V (BioRad) and stained with ethidium bromide for 20 min. PCR products were visualized under UV light on BioRad UV Transilluminator.

2.8. Real Time PCR

In order to carry out screening of 35S promoter, Real time PCR was conducted by CONGEN GMO screening kit in ABI 7500 Real Time PCR instrument. The solutions of GMO screening kit were briefly centrifuged in Hettich Micro 12-24.

PCR reaction mix components were collected in sterile 96 wells plates for both 35S promoter and NOS terminator real-time PCR studies. For these screening studies only the reagents within the CONGEN kit are used according to the procedure provided by the manufacturer. 17ul 35S/NOS reaction mix respectively, 1 ul FDE, 0.1 ul Taq DNA polimerase and 2 ul DNA for each reaction.

Cycling condition for real-time PCRs was illustrated in Table 2.13.
Table 2.13 Real-time PCR conditions for 35S promoter and NOS terminator regions.

<table>
<thead>
<tr>
<th>PCR programme</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>15 sec.</td>
</tr>
<tr>
<td>Extension</td>
<td>65°C</td>
<td>32 sec.</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

Number of cycles: 45
CHAPTER III

RESULTS AND DISCUSSION

3.1 DNA Isolation for GMO Detection Analysis

Quality and purity of nucleic acids are the most critical factors in meaningful PCR analysis. Therefore, it is very important to obtain pure and high quality DNA for our experiments. In this study CTAB DNA isolation method was used and also the extraction kits are preferred for the real time PCR analysis. CTAB method has been known as a suitable protocol for plant tissues for the elimination of polysaccharides and polyphenolic compounds.

Like many other plant species, tomato tissues contain high levels of polyphenolic compounds. When cells are disrupted, these compounds can come into contact with nuclei and other organelles. In their oxidized forms, polyphenols covalently bind to DNA giving it a brown color and making it useless for most research applications (Peterson et al., 1997). At the beginning of this thesis study, contamination of extracted DNAs from fresh tomatoes with polyphenolic compounds, caused inhibition of PCR amplification reactions. In order to increase the yield, CTAB protocol was modified by adding 1% PVP (Doyle & Doyle, 1990). However, in some cases especially for the extraction particularly from fresh tomato samples very efficient results are not obtained by using this protocol. DNA extracted using extraction kits gave more suitable results. Therefore, extraction kits are preferred for extraction of DNAs from some fresh tomato samples.
After isolation step, DNA samples were checked by running on the gel and by measuring the absorbance with spectrophotometer. DNA concentrations and obtained A260/ A280 values were summarized in Table 3.1 and Table 3.2. The reason for differences in concentration values was probably due to handling during sample homogenization with liquid nitrogen. A260/A280 value below 1.6, shown in samples FE1 and FE4, indicates a contamination with proteins and aromatic substances (Pauli et al., 2000), these two fresh tomato samples DNAs exhibited a critical A260/ A280 value were checked with all our DNAs in patatin PCR to control their amplification capacity. However, they gave acceptable results from these PCR studies, therefore they are used for further PCR analysis.
Table 3.1 Concentration of seed samples DNAs according to the spectrophotometer and their cultivar types.

<table>
<thead>
<tr>
<th>Sample No</th>
<th>WL: 260</th>
<th>WL: 280</th>
<th>RATIO</th>
<th>Concentration</th>
<th>cultivar</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE1Kapadokya</td>
<td>0.098</td>
<td>0.058</td>
<td>1.69</td>
<td>980 ng/ul</td>
<td>-</td>
</tr>
<tr>
<td>SE2</td>
<td>0.02</td>
<td>0.012</td>
<td>1.67</td>
<td>200 ng/ul</td>
<td>hybrid</td>
</tr>
<tr>
<td>SE3</td>
<td>0.0014</td>
<td>0.008</td>
<td>1.75</td>
<td>14 ng/ul</td>
<td>Rio grande</td>
</tr>
<tr>
<td>SE4</td>
<td>0.028</td>
<td>0.016</td>
<td>1.75</td>
<td>280 ng/ul</td>
<td>cherry</td>
</tr>
<tr>
<td>SE5</td>
<td>0.065</td>
<td>0.04</td>
<td>1.63</td>
<td>650 ng/ul</td>
<td>cherry</td>
</tr>
<tr>
<td>SE6</td>
<td>0.09</td>
<td>0.05</td>
<td>1.8</td>
<td>900 ng/ul</td>
<td>cherry</td>
</tr>
<tr>
<td>SE7</td>
<td>0.02</td>
<td>0.012</td>
<td>1.67</td>
<td>200 ng/ul</td>
<td>rio grande</td>
</tr>
<tr>
<td>SE8</td>
<td>0.04</td>
<td>0.023</td>
<td>1.74</td>
<td>400 ng/ul</td>
<td>falcon</td>
</tr>
<tr>
<td>SE9</td>
<td>0.037</td>
<td>0.019</td>
<td>1.95</td>
<td>370 ng/ul</td>
<td>invictus</td>
</tr>
<tr>
<td>SE10</td>
<td>0.064</td>
<td>0.039</td>
<td>1.64</td>
<td>640 ng/ul</td>
<td>falcon</td>
</tr>
<tr>
<td>SE11</td>
<td>0.032</td>
<td>0.018</td>
<td>1.78</td>
<td>320 ng/ul</td>
<td>sc2121</td>
</tr>
<tr>
<td>SE12</td>
<td>0.089</td>
<td>0.052</td>
<td>1.71</td>
<td>890 ng/ul</td>
<td>h2274</td>
</tr>
<tr>
<td>SE13</td>
<td>0.084</td>
<td>0.048</td>
<td>1.75</td>
<td>840 ng/ul</td>
<td>cherry</td>
</tr>
<tr>
<td>SE14</td>
<td>0.16</td>
<td>0.1</td>
<td>1.6</td>
<td>1600 ng/ul</td>
<td>rio grande</td>
</tr>
<tr>
<td>SE15</td>
<td>0.018</td>
<td>0.011</td>
<td>1.64</td>
<td>180 ng/ul</td>
<td>rio grande</td>
</tr>
<tr>
<td>SE16</td>
<td>0.084</td>
<td>0.045</td>
<td>1.86</td>
<td>840 ng/ul</td>
<td>cherry</td>
</tr>
<tr>
<td>SE17</td>
<td>0.056</td>
<td>0.032</td>
<td>1.75</td>
<td>560 ng/ul</td>
<td>Falcon</td>
</tr>
<tr>
<td>SE18</td>
<td>0.092</td>
<td>0.054</td>
<td>1.7</td>
<td>920 ng/ul</td>
<td>H2274</td>
</tr>
<tr>
<td>SE19</td>
<td>0.096</td>
<td>0.055</td>
<td>1.75</td>
<td>960 ng/ul</td>
<td>H2274</td>
</tr>
<tr>
<td>SE20</td>
<td>0.13</td>
<td>0.07</td>
<td>1.86</td>
<td>1300 ng/ul</td>
<td>Cherry</td>
</tr>
<tr>
<td>SE21</td>
<td>0.27</td>
<td>0.15</td>
<td>1.8</td>
<td>2700 ng/ul</td>
<td>Cherry</td>
</tr>
<tr>
<td>SE22</td>
<td>0.039</td>
<td>0.02</td>
<td>1.95</td>
<td>390 ng/ul</td>
<td>Rio grande</td>
</tr>
<tr>
<td>SE23</td>
<td>0.041</td>
<td>0.022</td>
<td>1.86</td>
<td>410 ng/ul</td>
<td>invictus</td>
</tr>
<tr>
<td>SE24</td>
<td>0.03</td>
<td>0.017</td>
<td>1.76</td>
<td>300 ng/ul</td>
<td>H2274</td>
</tr>
<tr>
<td>SE25</td>
<td>0.069</td>
<td>0.04</td>
<td>1.73</td>
<td>690 ng/ul</td>
<td>Hybrid</td>
</tr>
<tr>
<td>SE26</td>
<td>0.081</td>
<td>0.046</td>
<td>1.76</td>
<td>810 ng/ul</td>
<td>H2274</td>
</tr>
<tr>
<td>SE27</td>
<td>0.043</td>
<td>0.022</td>
<td>1.95</td>
<td>430 ng/ul</td>
<td>Sc2121</td>
</tr>
<tr>
<td>SE28</td>
<td>0.072</td>
<td>0.041</td>
<td>1.76</td>
<td>720 ng/ul</td>
<td>Cherry</td>
</tr>
<tr>
<td>SE29</td>
<td>0.084</td>
<td>0.05</td>
<td>1.68</td>
<td>840 ng/ul</td>
<td>Rio grande</td>
</tr>
<tr>
<td>SE30</td>
<td>0.066</td>
<td>0.039</td>
<td>1.69</td>
<td>660 ng/ul</td>
<td>invictus</td>
</tr>
<tr>
<td>SE31</td>
<td>0.021</td>
<td>0.012</td>
<td>1.75</td>
<td>210 ng/ul</td>
<td>-</td>
</tr>
<tr>
<td>SE32</td>
<td>0.019</td>
<td>0.010</td>
<td>1.93</td>
<td>190 ng/ul</td>
<td>-</td>
</tr>
<tr>
<td>SE33</td>
<td>0.09</td>
<td>0.051</td>
<td>1.78</td>
<td>900 ng/ul</td>
<td>cherry</td>
</tr>
<tr>
<td>PSG</td>
<td>0.033</td>
<td>0.017</td>
<td>1.94</td>
<td>330 ng/ul</td>
<td>cherry</td>
</tr>
<tr>
<td>PSC</td>
<td>0.055</td>
<td>0.032</td>
<td>1.72</td>
<td>550 ng/ul</td>
<td>-</td>
</tr>
<tr>
<td>Sample No:</td>
<td>Sample ID:</td>
<td>WL:260</td>
<td>WL:280</td>
<td>RATIO</td>
<td>Concentration</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
<td>---------------</td>
</tr>
<tr>
<td>FE1</td>
<td>Kapadokya</td>
<td>0.089</td>
<td>0.058</td>
<td>1.5</td>
<td>890 ng/ul</td>
</tr>
<tr>
<td>FE2</td>
<td>Antalya real</td>
<td>0.031</td>
<td>0.015</td>
<td>2.06</td>
<td>310 ng/ul</td>
</tr>
<tr>
<td>FE3</td>
<td>Muğla</td>
<td>0.093</td>
<td>0.047</td>
<td>1.98</td>
<td>930 ng/ul</td>
</tr>
<tr>
<td>FE4</td>
<td>İzmir</td>
<td>0.065</td>
<td>0.043</td>
<td>1.51</td>
<td>650 ng/ul</td>
</tr>
<tr>
<td>FE5</td>
<td>Bartın</td>
<td>0.014</td>
<td>0.079</td>
<td>1.78</td>
<td>140 ng/ul</td>
</tr>
<tr>
<td>FE6</td>
<td>Mersin</td>
<td>0.016</td>
<td>0.09</td>
<td>1.78</td>
<td>160 ng/ul</td>
</tr>
<tr>
<td>FE7</td>
<td>Ayaş</td>
<td>0.027</td>
<td>0.016</td>
<td>1.69</td>
<td>270 ng/ul</td>
</tr>
<tr>
<td>FE8</td>
<td>Antalya cherry</td>
<td>0.024</td>
<td>0.014</td>
<td>1.71</td>
<td>240 ng/ul</td>
</tr>
<tr>
<td>FE9</td>
<td>Ayvalık1</td>
<td>0.035</td>
<td>0.016</td>
<td>2.19</td>
<td>350 ng/ul</td>
</tr>
<tr>
<td>FE10</td>
<td>Ayvallık2</td>
<td>0.037</td>
<td>0.017</td>
<td>2.17</td>
<td>370 ng/ul</td>
</tr>
<tr>
<td>FE11</td>
<td>Ayvalık3</td>
<td>0.01</td>
<td>0.006</td>
<td>1.67</td>
<td>100 ng/ul</td>
</tr>
<tr>
<td>FE12</td>
<td>Yunanistan</td>
<td>0.026</td>
<td>0.013</td>
<td>2</td>
<td>260 ng/ul</td>
</tr>
<tr>
<td>FE13</td>
<td>Dikmen</td>
<td>0.031</td>
<td>0.016</td>
<td>1.94</td>
<td>310 ng/ul</td>
</tr>
<tr>
<td>FE14</td>
<td>Gölbasi</td>
<td>0.015</td>
<td>0.007</td>
<td>2.14</td>
<td>150 ng/ul</td>
</tr>
<tr>
<td>FE15</td>
<td>Eryaman1</td>
<td>0.091</td>
<td>0.05</td>
<td>1.82</td>
<td>910 ng/ul</td>
</tr>
<tr>
<td>FE16</td>
<td>Eryaman2</td>
<td>0.014</td>
<td>0.008</td>
<td>1.75</td>
<td>140 ng/ul</td>
</tr>
<tr>
<td>FE17</td>
<td>Balgat</td>
<td>0.084</td>
<td>0.044</td>
<td>1.91</td>
<td>840 ng/ul</td>
</tr>
<tr>
<td>FE18</td>
<td>Antalya p1</td>
<td>0.072</td>
<td>0.04</td>
<td>1.8</td>
<td>720 ng/ul</td>
</tr>
<tr>
<td>FE19</td>
<td>Antalya p2</td>
<td>0.093</td>
<td>0.049</td>
<td>1.9</td>
<td>930 ng/ul</td>
</tr>
<tr>
<td>FE20</td>
<td>Antalya cherry2</td>
<td>0.044</td>
<td>0.021</td>
<td>2.09</td>
<td>440 ng/ul</td>
</tr>
<tr>
<td>FE21</td>
<td>Nallihan</td>
<td>0.071</td>
<td>0.043</td>
<td>1.65</td>
<td>710 ng/ul</td>
</tr>
<tr>
<td>FE22</td>
<td>Eskişehir1</td>
<td>0.086</td>
<td>0.049</td>
<td>1.76</td>
<td>860 ng/ul</td>
</tr>
<tr>
<td>FE23</td>
<td>Eskişehir2</td>
<td>0.065</td>
<td>0.042</td>
<td>1.55</td>
<td>650 ng/ul</td>
</tr>
<tr>
<td>FE24</td>
<td>Antalya1</td>
<td>0.027</td>
<td>0.015</td>
<td>1.8</td>
<td>270 ng/ul</td>
</tr>
<tr>
<td>FE25</td>
<td>Antalya2</td>
<td>0.062</td>
<td>0.038</td>
<td>1.63</td>
<td>620 ng/ul</td>
</tr>
<tr>
<td>FE26</td>
<td>Antalya3</td>
<td>0.081</td>
<td>0.044</td>
<td>1.84</td>
<td>810 ng/ul</td>
</tr>
<tr>
<td>FE27</td>
<td>Antalya4</td>
<td>0.04</td>
<td>0.022</td>
<td>1.82</td>
<td>400 ng/ul</td>
</tr>
<tr>
<td>FE28</td>
<td>Antalya5</td>
<td>0.039</td>
<td>0.02</td>
<td>1.95</td>
<td>390 ng/ul</td>
</tr>
<tr>
<td>FE29</td>
<td>Antalya6</td>
<td>0.079</td>
<td>0.046</td>
<td>1.72</td>
<td>790 ng/ul</td>
</tr>
<tr>
<td>FE30</td>
<td>Antalya7</td>
<td>0.036</td>
<td>0.019</td>
<td>1.89</td>
<td>360 ng/ul</td>
</tr>
<tr>
<td>FE31</td>
<td>Çanakkale1</td>
<td>0.055</td>
<td>0.03</td>
<td>1.83</td>
<td>550 ng/ul</td>
</tr>
<tr>
<td>FE32</td>
<td>Çanakkale2</td>
<td>0.021</td>
<td>0.012</td>
<td>1.75</td>
<td>210 ng/ul</td>
</tr>
<tr>
<td>FE33</td>
<td>Çanakkale3</td>
<td>0.054</td>
<td>0.029</td>
<td>1.86</td>
<td>540 ng/ul</td>
</tr>
<tr>
<td>FE34</td>
<td>İstanbul ischer</td>
<td>0.033</td>
<td>0.019</td>
<td>1.74</td>
<td>330 ng/ul</td>
</tr>
<tr>
<td>FE35</td>
<td>Balgat</td>
<td>0.049</td>
<td>0.027</td>
<td>1.81</td>
<td>490 ng/ul</td>
</tr>
<tr>
<td>FE36</td>
<td>Bilkent</td>
<td>0.091</td>
<td>0.05</td>
<td>1.82</td>
<td>910 ng/ul</td>
</tr>
<tr>
<td>FE37</td>
<td>İstanbul yer</td>
<td>0.017</td>
<td>0.009</td>
<td>1.89</td>
<td>170 ng/ul</td>
</tr>
<tr>
<td>FE38</td>
<td>Fethiye</td>
<td>0.021</td>
<td>0.13</td>
<td>1.62</td>
<td>210 ng/ul</td>
</tr>
<tr>
<td>FE39</td>
<td>Trabzon</td>
<td>0.034</td>
<td>0.019</td>
<td>1.79</td>
<td>340 ng/ul</td>
</tr>
<tr>
<td>FE40</td>
<td>Trabzon</td>
<td>0.04</td>
<td>0.022</td>
<td>1.81</td>
<td>400 ng/ul</td>
</tr>
</tbody>
</table>
3.2 PCR Amplifications

In order to control, screen and construct specific PCR, 20 different primer sets were tried and thirteen of them were reported with each sample. Eight of the PCR systems were developed for the detection of transgenic elements commonly used in genetically modified crops, three different primers were used to identify the traits and two of the primer sets were used to perform construct specific PCR analysis.

3.2.1 Patatin Specific Control PCR

Pat-F/Pat-R primer set was used to check whether isolated DNA samples are corresponding to a tomato or not and to determine the amplification capacity of the tomato DNA. All our fresh tomato samples and seed samples were tested with this PCR system. In order to avoid the arising of false negative result due to the presence of PCR inhibitors like proteins, polysaccharides and polyphenolic compounds, it is highly recommended to perform a control experiment to test PCR inhibitors. Studies of (Jaccaud et al., 2003) have shown that potato, tomato and tobacco DNA’s generated a 124 bp amplified whereas no fragments were amplified from soya, maize, and rapeseed samples. Therefore, these primers have been found to be specific for Solanacea (Jaccaud et al., 2003), the botanical family to which tomato, tobacco, and potato are belonging.

The samples which did not produce 124 bp amplicon are eliminated and extraction is repeated until pure DNA obtained from these tomato samples. Otherwise, false positive or negative results would be inevitable.

As a result, except the maize and soy DNAs which were used as negative control, all other samples showed a very intense 124 bp amplicon that indicates their
amplification capacity for further detection analysis. In figure 3.1, 124 bp amplicons produced from some tomato and seed samples DNAs were shown.

**Figure 3.1 Patatin specific PCR results:** line1:M1; 100bp molecular weight marker, line 2: no-template, line3: maize DNA, line4-39 tomato and seed DNA samples, Line40:M2; 100bp molecular weight marker.

### 3.2.2 Screening PCR Systems for the Detection of GM Tomatoes

By using different primer sets, it was aimed to detect three screening targets that are commonly used in GMO detection that are 35S promoter, NOS terminator, kanamycin resistance marker gene. In detection studies, such methods are accepted only as an indication of presence of a genetic modification, and the presence of such targets should be further assessed by specific PCR detection methods. The antibiotic resistance gene NptII derived from *Escherichia coli* (Bruderer & Leitner, 2003), and regulatory elements such as the NOS terminator from *Agrobacterium tumefaciens* (Lin *et al.*, 2000), and the 35S promoter from the Cauliflower mosaic virus (Lin *et al.*, 2000) have been used for general screening of genetically modified crops especially for transgenic tomato lines as these regulatory elements are inserted to the
authorized transgenic tomato lines. However, 35S CaMV promoter, NOS terminator and kanamycin resistance marker gene were not used in all of the transgenic lines that were developed. Different promoters, terminators and markers could be used for the development of transgenic lines. Therefore, it should be notified that these targets are not necessarily being the main targets for detection of all GM plants.

All positive PCR products obtained from the all screening and identification studies were run again by gel electrophoresis and depicted from the Figure 3.2 to Figure 3.17. The gel photos corresponding to both negatives and positives were shown in Appendix N.

3.2.2.1 CaMV-35S Promoter specific PCR system

One of the most important factors for achieving the desired expression levels of a transgene is the choice of the promoter that regulates transcription of the transgene. Many of the approved transgenic crops contain a copy of the constitutive 35S promoter (P-35S) from the CaMV or one of the derivatives of this promoter like the enhanced and duplicated 35S promoter regions. The P-35S has been widely used in the screening detection methods. At total 56% of genetically modified crops contains CaMV35S promoter which provides constitutive expression of inserted gene of interest (Bruderer & Leitner, 2003).

In this study, four different primer sets for the detection of 35S promoter are tried. However two of them gave accurate results. Three of them were from the literature and one of them was designed for this study. 35S cf3/cr4 primer set (Lipp et al, 2001) amplifying 123 bp amplicon did not work well in our studies. Even gradient PCR optimization was carried out; smears were not dissappeared so that clear bands could not be obtained for any samples even the positive samples (data not shown).
Validated primer set 35S-f/35S-r that allows the amplification of a 195 bp fragment from the 35S promoter of the cauliflower mosaic virus (P-35S) was also used. In this PCR system amplification signal was observed in seven of seed samples and nine of fresh tomato samples and also in our positive controls (Figure 3.2).

P35S-aflu/P35S-ar1 primer set (Wolf et al., 1999) produced very clear 207 bp amplicon with our positive samples and when these results were sequenced, gave homology with the sequence of NCBI Gene Bank accession no: AF078810 (Figure 3.3). However, the results were not reproducible when the PCR analysis was repeated with the same samples in the same conditions. Therefore, a new primer set is designed in order to screen 35S promoter region.

![Figure 3.2 35S Promoter specific positive PCR results by using 35S f/r primer set: line1: SE1, line2: SE3, line3: SE9, line4: SE11, line5: SE13, line6: SE19, line7: SE20, line8: SE27, line9: SE31, line10: FE9, line11: FE11, line12: FE15, line13: FE16, line14: FE20, line15: FE26, line16: FE27, line17: FE29, line18: FE32, line19: FE34, line20: no-template, line21: 100 bp ladder plus.](image-url)
Figure 3.3 35S Promoter specific positive PCR results using P35S-af1u/P35S-ar1 primer set: line1: 100 bp ladder, line2: SE1, line3: no-template, line4: SE3, line5: SE9, line6: SE11, line7: SE13, line8: SE19, line9: SE27, line10: SE31, line11: FE9, line12: FE11, line13: FE20, line14: FE27, line15: FE29, line16: FE32, line17: PSG.

The primer set 35S-EUF/35S-EUR was designed against the 35S promoter sequence with an NCBI GenBank accession no. AF078810.

35S EUF/R primer set amplified 392 bp amplicon with eight seed samples and ten fresh tomato samples and also in our positive controls (Figure 3.4). This primer set produced accurate and reproducible results.

Table 3.3 Sequences Producing Significant Alignments with 35S specific PCR products using P3SS-af1u/P3SS-ar1 primer set

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E value</th>
<th>Max ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF565885.1</td>
<td>pDuExB2, complete sequence</td>
<td>292</td>
<td>292</td>
<td>89%</td>
<td>2e-76</td>
<td>99%</td>
</tr>
<tr>
<td>EF565884.1</td>
<td>pDuExB, complete sequence</td>
<td>292</td>
<td>292</td>
<td>89%</td>
<td>2e-76</td>
<td>99%</td>
</tr>
</tbody>
</table>
Alignment of 35S Promoter Specific PCR product obtained using P35S-aflu/P35S-ar1 with plant expression vectors 35s promoter region.

>gb|EF565885.1| Plant expression vector pDuExB2 (pDuExD7), complete sequence
Length = 8643
Score = 292 bits (158), Expect = 2e-76
Identities = 161/162 (99%), Gaps = 1/162 (0%)
Strand = Plus/Plus

Sample SE19

16
AGATGCTCTGCCGCAT-GTTGCCAAGATGGACCCACCCACAGGAGCATTGGA 74
35s promoter region of vector
6223
AGATGCTCTGCGCACAGTGTTGCCAAGATGGACCCACCCACAGGAGCATTGGA 6282

Sample SE19

75
AAAAGAGAGACGTTCCAACCACGCTTTCAAAGCAAGTGATTTAGATGTATATCTCCACTGA 134
35s promoter region of vector
6283
AAAAGAGAGACGTTCCAACCACGCTTTCAAAGCAAGTGATTTAGATGTATATCTCCACTGA 6342

Sample SE19

135
CGTAAGGGGATGACGCGACAATCCCATATCTTCCGCARGGACCC 176
35s promoter region of vector
6343
CGTAAGGGGATGACGCGACAATCCCATATCTTCCGCARGGACCC 6384
Table 3.4 Sequences Producing Significant Alignments with 35S specific PCR products using 35SEUF/35SEUR primer set

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E value</th>
<th>Max ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF565885.1</td>
<td>pDuExB2, complete sequence</td>
<td>555</td>
<td>555</td>
<td>34%</td>
<td>1e-154</td>
<td>98%</td>
</tr>
<tr>
<td>EF565884.1</td>
<td>pDuExB, complete sequence</td>
<td>555</td>
<td>555</td>
<td>34%</td>
<td>1e-154</td>
<td>98%</td>
</tr>
</tbody>
</table>

Alignment of 35S promoter specific PCR Product obtained using 35S EUF/EUR primer set with plant expression vectors 35S Promoter region

>gb|EF565885.1| Plant expression vector pDuExB2 (pDuExD7), complete sequence
Length=8643
Score = 555 bits (300), Expect = 1e-154
Identities = 309/313 (98%), Gaps = 2/313 (0%)
Strand=Plus/Plus

SAMPLE FE15
39
GAAGACC--AAGAGCAAATGGAGACTTTTC--ACAAAGGGTTAATATCCGAAAAACCTCCCTCGGA 96
|
|
35S promoter expected sequence
6062
GAAGACCAAGGGCAATTGGAGACTTTTCACAACAAAAGGGTTAATATCCGAAAAACCTCCCTCGGA 6121

SAMPLE FE15
97
TTCCATTGCCAGCTATCTGTCACTTTATTTGGAAGATAGTGGAATAGGAAGAAGTGGCCTCC 156
|
|
35S promoter expected sequence
6122
TTCCATTGCCAGCTATCTGTCACTTTATTTGGAAGATAGTGGAATAGGAAGAAGTGGCCTCC 6181
SAMPLE FE15
157
TACAAAAATGCCATCATTTCCAGATAAAGGGAGGAGCAGCTCTCCGACGACGAT 216
35s promoter expected sequence
6182
TACAAAAATGCCATCATTTCCAGATAAAGGGAGGAGCAGCTCTCCGACGACGAT 6241

SAMPLE FE15
217
GGTTCCAAAGATCGACCCCACCACCGACGCTGCTCGTAAGAGAGACGTTCCACCCAC 276
35s promoter expected sequence
6242
GGTTCCAAAGATCGACCCCACCACCGACGCTGCTCGTAAGAGAGACGTTCCACCCAC 6301

35sre 277
ACGTCTTTCACAAAGCAATGACTGATGTATCTCCACTGACGTAAGGGATGACGCACAA 336
35s promoter expected sequence 6302
ACGTCTTTCACAAAGCAATGACTGATGTATCTCCACTGACGTAAGGGATGACGCACAA 6361

SAMPLE FE15
337
TCCCTCTTCCTT 349
35s promoter expected sequence
6362
TCCCTCTTCCTT 6374

3.2.2.2. NOS Terminator specific PCR system

The NOS terminator (T-NOS), isolated from the nopaline synthase gene of *Agrobacterium tumefaciens* is commonly used terminator in transgenic crops for the termination of transcript of trait genes. 37% of approved genetically modified crops contain *A. tumefaciens* NOS terminator region (Bruderer & Leitner, 2003).

The validated primer set Nos-1/Nos-3 allows the amplification of a 180 bp fragment. In 17 samples, 180 bp amplicon indicating the presence of NOS terminator region
were observed (Figure 3.5). In this Figure, the positive results were shown after running the positive PCR results in gel electrophoresis.

Another primer designed for detection of NOS terminator region was designed. This primer set amplify 207 bp amplicon in samples containing NOS terminator region. In 11 seed and 5 fresh samples, 207 bp amplicon was observed (Figure 3.6, 3.7). PCR products were sequenced by lontek (Istanbul) sequencing analysis results showed that these primers amplify NOS region. (Figure 3.8)

![Figure 3.5 NOS terminator specific positive PCR results using nosf/r primer set: line1: 100 bp ladder, line2: SE1, line3: SE3, line4: SE6, line5: SE9, line6: SE11, line7: SE13, line8: SE15, line9: SE17, line10: SE20, line11: SE26, line12: SE30, line13: FE11, line14: FE15, line15: FE26, line16: FE27, line17: FE34, line18: Bt11, line19: PSG, line20: no-template, line21: no-template, line22: 100 bp ladder.](image-url)
Figure 3.6 NOS terminator specific positive PCR results for seed samples using nos207f/r primer set: line1: 100 bp ladder, line2: PSG, line3: SE3, line4: SE6, line5: SE9, line6: SE11, line7: SE13, line8: SE15, line9: SE17, line10: SE19, line11: SE20, line12: SE26, line13: SE30, line14: ROKII plasmid, line15: Bt11, line16: No-template.

Figure 3.7 NOS terminator specific positive PCR results for fresh samples using nos207f/r primer set: line1: ROKII plasmid, line2: FE11, line3: FE15, line4: FE26, line5: FE27, line6: FE34, line7: PSC, line8: No-template, line9: 100 bp ladder.
Figure 3.8 ClustalW analysis result of the obtained NOS Terminator sequence using NOS 207 F/R primer set and the U12540 Pbin19 NOS terminator sequence (*, indicates the homologous base pairs).

3.2.2.3 NptII gene specific PCR system

More than 40 distinct genes have been used for generation of currently approved transgenic crops. The most frequently used transgene is NptII, originating from the E.coli transposon 5. NptII gene is used as a marker gene in the construction of transgenic plants; the expression of this gene confers resistance to the antibiotic kanamycin. The purpose of inserting the NptII gene into crops with any other
transgene is to have an effective method for indicating the success of genetic modification.

In screening of genetically modified tomatoes, detecting the NptII gene has an extraordinary importance. Nearly all approved genetically modified tomato lines including 1345-4, 35 1 N, 5345, 8338, B-Da-F and Flavr Savr contains NptII kanamycin resistance gene (Bruderer & Leitner, 2003) (Table 3.4). Although the three regions; 35S promoter, NOS terminator and NptII are the most widely used screening targets, kanamycin resistance gene provides the most general detection for tomato among those regions. It gives the opportunity to screen different GM tomatoes with different modified characteristics like delayed ripening and insect resistance.

Therefore, the tomato samples were tested for the presence of NptII gene by means of the primer pairs Kan-F/Kan-R, kan411f/r, TN5f/r which generate an amplicon of 459 bp, 411 bp and 175 bp in length, respectively. 11 seed samples and 8 fresh tomato samples produced the expected 459 bp amplification signal (Figure 3.9, 3.10, 3.11).

Resulted PCR products were sequenced by Iontek (Istanbul). Sequencing analysis results showed that these primers amplify kanamycin resistance gene region. (Figure 3.12, 3.13)

Table 3.5 Sequences Producing Significant Alignments with kanamycin resistance gene-specific PCR products using kan F/R primer set

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E value</th>
<th>Max ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ813654.2</td>
<td>Cloning vector pINT, complete sequence</td>
<td>728</td>
<td>728</td>
<td>88%</td>
<td>0.0</td>
<td>99%</td>
</tr>
<tr>
<td>AB294467.1</td>
<td>Gateway binary vector pGWB455 DNA, complete sequence</td>
<td>728</td>
<td>728</td>
<td>88%</td>
<td>0.0</td>
<td>99%</td>
</tr>
<tr>
<td>AB294466.1</td>
<td>Gateway binary vector pGWB454 DNA, complete sequence</td>
<td>728</td>
<td>728</td>
<td>88%</td>
<td>0.0</td>
<td>99%</td>
</tr>
</tbody>
</table>
Alignment of NPTII Specific PCR product obtained using kanf/r primer set with cloning vectors kanamycin resistance gene region.

AF274974_expected_sequence
SE9
CGGCTTCTTTTGTCAAGACCGACCCTGTCGGTGCTGATAGAAGACTGCA

AF274974_expected_sequence
SE9
GGACGGGACGAGCCGGGCTATGCGGGGAGCCGAGGGCTCTTTGCG

AF274974_expected_sequence
SE9
CAGCTGCTGCAGCTGGCTGAGAGCCGAGGGCCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
GGCGAGGTGCCGGCGCGATACTCCGCACCTGAACGTCCGAGGGCAGGGCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
GAACTGCTCACTCAGCGACGGGTGGCTCGATGGGGGAGGGCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
CAGCTGCTGCAGCTGGCTGAGAGCCGAGGGCCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
GGCGAGGTGCCGGCGCGATACTCCGCACCTGAACGTCCGAGGGCAGGGCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
GAACTGCTCACTCAGCGACGGGTGGCTCGATGGGGGAGGGCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
GGCGAGGTGCCGGCGCGATACTCCGCACCTGAACGTCCGAGGGCAGGGCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
GAACTGCTCACTCAGCGACGGGTGGCTCGATGGGGGAGGGCGAGGGCTCTTTG

AF274974_expected_sequence
SE9
GGCGAGGTGCCGGCGCGATACTCCGCACCTGAACGTCCGAGGGCAGGGCGAGGGCTCTTTG

Figure 3.10 NPTII specific positive PCR results using kan411f/r primer set:
line1: 100 bp ladder, line2: SE1, line3: SE3, line4: SE6, line5: SE9, line6: SE11,
Figure 3.11 ClustalW analysis result of the obtained NptII sequence using kan411fl/411r primer set and the target AF274974 sequence (*, indicates the homologous base pairs).

NPTII specific PCR results using TN5f/r primer set were not sequenced as PCR products were too short in length to analyze.

3.2.3 Identification PCR Systems Specific to Ripening Delayed Tomatoes

3.2.3.1 Identification of Sam-k Gene

Sam3F/5F primer set producing 217 bp amplicon was used in order to identify sam-k gene. Sample SE17 was previously screened for sam-k gene and found positive by Turkoglu in 2007 in our laboratory. This sample was used as positive control for sam-k gene specific detection studies. According to the sam-k specific PCR results,
all of the tomato samples were sam-k negative except the seed sample SE17 which was previously screened by Turkoglu (2007).

Figure 3.13 Sam-k specific positive PCR results using sam3f/5f primer set: line1: no template, line3: SE17, line 11: 100 bp ladder, other lines are some of the sam-k negative tomato samples.

3.2.3.2 Identification of PG Gene

In order to control if there were any F type ripening delayed tomato in our samples, PG34F/R primer set (Meyer, 1995) was used. As truncated polygalacturonase coding gene has been inserted to the F type ripening delayed tomatoes, 180 bp amplicon is observed in transgenic lines, whereas non-transgenic lines give 380 bp amplicon. All of the samples produced 380 bp but none of them 180 bp. Therefore, it can be said that there were not any F type ripening delayed tomatoes or tomato seeds among the analyzed samples (Figure3.14). Although these samples were not F type ripening delayed transgenic tomatoes, they might be related to different types of ripening delayed GM lines such as B, Da, 1345-4,8338 or Flavr Savr lines. According to the data obtained from the literature even Flavr Savr transgenic tomatoes which carries
the antisense PG gene can be identified using PCR methods. (http://www.bats.ch/bats/publikationen/1997-2_gmo/tab14_gmo_primer.php).


3.2.3.3 Identification PCR Systems Specific to Insect Resistant Tomatoes (Gene specific target system)

Cry genes from Bacillus thuringiensis are widely used genes in transgenic crops. The cry genes, used in GM crops are all synthetic and modified, in some cases truncated forms of native genes are used, in order to optimize gene expression in the
plants. There are some studies about insect resistant genetically modified tomatoes containing cry1Ab and cry1Ac genes in literature (Fischhoff et al., 1987; Delannay et al., 1989; Kumar & Kumar, 2004; Mandokar et al., 2000) but Monsanto’s transgenic line 5345-1 is the one that has an approval from FDA in USA (Bruderer & Leitner, 2003).

*Bacillus thuringiensis* subsp. *kurstaki* HD73 strain were found from NCBI Gene Bank. By comparing the natural cry1Ac and synthetic cry1Ac sequences with ClustalW homology search programme, a primer was designed that is specific to homologous regions between the natural cry1Ac and synthetic cry1Ac sequences. Synthetic cry1Ac sequences; U63372, AY525369 and natural cry1Ac sequences DQ285666, EF094884 were compared and a primer designed for their homologous region. Natural and synthetic cry1Ac gene sequence alignments can be seen in Appendix M.

Primer pair Cry323F / Cry323R was designed targeting the homologous regions of these four sequences. In primer design studies, it was observed that this newly synthesized primer set should produce 323 bp amplicon with cry1Ac sequence. Expected PCR amplification signals were observed in 7 seed and 11 fresh samples. (Figure 3.15)

In order to confirm the results of cry1Ac PCR products, firstly 30ml of PCR product was sent for automatic sequencing. After DNA purification from agarose gel, bands were sequenced with 10 mmole primer by IONTEK (Istanbul/Turkey). (Table 3.6)
Figure 3.15 Cry1Ac specific positive PCR results using CRY323F/R primer set:
line1: 100 bp ladder, line2: SE3, line3: SE6, line4: SE9, line5: SE13, line6: SE19, 
line7: SE27, line8: SE30, line9: FE9, line10: FE11, line11: FE20, line12: FE26, 
line13: FE27, line14: FE28, line15: FE29, line16: FE30, line25: FE32, line18: 
FE34, line19: FE35, line20: ECE4 (+ control plasmid), line 21: SE1 (- control), 22: 
no template, line 23: 100 bp ladder.

The obtained results from sequencing were compared with the expected cry1Ac 
sequences. The results of this comparison exhibited high homologies according to 
sequencing results, indicating that PCR products are related to the cry1Ac 
expressing gene (Table 3.6).
Table 3.6 Sequences Producing Significant Alignments with cry1Ac gene-specific PCR products using cry323f/r primer set

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E value</th>
<th>Max ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY525369.1</td>
<td>cry1AcAT modified toxin gene, complete cds Bacillus thuringiensis</td>
<td>244</td>
<td>244</td>
<td>16%</td>
<td>3e-61</td>
<td>97%</td>
</tr>
<tr>
<td>AY730621.1</td>
<td>plasmid Cry1Ac (cry1Ac) gene, complete cds Bacillus thuringiensis</td>
<td>244</td>
<td>244</td>
<td>16%</td>
<td>3e-61</td>
<td>97%</td>
</tr>
<tr>
<td>AY122057.1</td>
<td>insecticidal crystal protein Cry1Ac (cry1Ac) gene, complete cds</td>
<td>239</td>
<td>239</td>
<td>16%</td>
<td>2e-59</td>
<td>97%</td>
</tr>
</tbody>
</table>

Alignment of cry1Ac Specific PCR product obtained using Cry323f/r primer set with synthetic cry1Ac gene sequence.

```
>gb|AY525369.1| Synthetic construct cry1AcAT modified toxin gene, complete cd
Length=2028
Score = 244 bits (132), Expect = 3e-61
Identities = 138/141 (97%), Gaps = 0/141 (0%)
Strand=Plus/Plus

Query  266  AGGCTTATTATTATTGTCTACGCGCATCAAAATATGCTACGGACGCTGATAGCTCTGCTATTCTCTCTCGCTTCTGGGGGGG  325

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>..........................</td>
<td></td>
</tr>
</tbody>
</table>

Shbjct  931  AGGCGCTTATTATTATTGTCTACGCGCATCAAAATATGCTACGGACGCTGATAGCTCTGCTATTCTCTCTCGCTTCTGGGGGGG  990

Query  326  CCAATTCATACTTCTCTTACGATGTGGAAATGAATGCCATCCACACAAAGCTTATT  385

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>..........................</td>
<td></td>
</tr>
</tbody>
</table>

Shbjct  991  CCAATTCATACTTCTCTTACGATGTGGAAATGAATGCCATCCACACAAAGCTTATT  105

Query  386  GTTGCTCAACTAGGTCATGGG  406

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>..........................</td>
<td></td>
</tr>
</tbody>
</table>

Shbjct  1051  GTTGCTCAACTAGGTCATGGG  1071
```
3.2.4 Construct specific PCR Systems

3.2.4.1 Construct specific PCR Systems Specific to 5345-1 line

In order to ensure that the presence of synthetic cry1Ac rather than natural cry1Ac, construct specific PCR system was designed. For this purpose cry1Ac gene carrying samples were used and the 5345-1 line was targetted. According to the genetic map of 5345-1 line, it is known that cry1Ac is near the pE-35S promoter (Figure 1.2). Construct specific primer sets were designed for the junction region in between the cry1Ac gene and the pE-35S promoter. The expected amplification product was nearly 700 bp for Crycsf/Crycsr primer set and 400 bp for crycsf/crycsr primer set. Although crycsf/r primer set do not produce any amplicon, crycsf/r primer sets produces intense bands. However, unspecific bands were not eliminated eventhough gradient PCR was used. In order to anlyze the sequence of PCR products, removal of unspecific amplicons was highly needed. For this purpose, gel extraction kit (Fermentas) was used according to the manufacturer’s instructions.
Figure 3.16 Construct Specific PCR results specific to 5345-1 line using Cryesf/Crycsr primer set: line1: no template, line2: SE6, line3: FE19, line4: 100 bp ladder.

Although an amplicon was produced by using construct specific primers, the sequence analysis results did not show homology with the expected construct. Sequence analysis results were evaluated by using BLAST analysis programme to in order to analyze the sequence of the construct specific PCR products. The results obtained from the BLAST analysis demonstrated that the construct specific PCR products sequences show high homology with the genomic DNA of the *Lycopersicon esculentum*. Such kind of result might be caused from the unspecificity of the primers producing tomato genomic DNA. Another possible reason for such result that suspected tomato samples might be related to different kinds of transgenic insect resistant tomato lines from the 5345-1 line. Therefore, the expected construct was not exist in the analyzed samples genomes.
3.2.4.2 Construct specific PCR Systems Specific to 35 1 N line

In order to verify the 35 1 N line, construct specific PCR was carried out. For this purpose sam-k gene carrying seed sample SE17 was used and the 35 1 N line was targetted. According the genetic map of 35 1 N line sam-k gene is near the NOS terminator region (Figure1.5). Construct specific primer sets were designed for the junction region in between the sam-k gene and the NOS terminator. The expected amplicon length was nearly 400 bp.

The expected amplicon was obtained from the construct specific PCR studies (Figure 3.17). PCR products were separated from the unspecific amplicons by using Fermentas gel extraction kit according to the manufacturer’s instructions then sent for sequencing. Sequence analysis results shows 60% homology with the expected construct sequence. (Figure 3.18)

![Figure 3.17 Construct Specific PCR results specific to 35 1 N line using sameuf/sameur primer set: line1-10: SE17, line11: 1 kb ladder.](image)
Figure 3.18 ClustalW analysis result of the obtained construct specific sam-k-nos junction sequence using sameulf/r primer set. (*, indicates the homologous base pairs).

3.2.5 Real time PCR Systems Specific to 35S promoter and NOS terminator

Real-Time PCR ABI7500 instrument and CONGEN qualitative GMO detection kit were used in order to verify conventional 35S promoter and NOS terminator positive samples. Results are shown in Figures 3.19, 3.20, 3.21 and 3.22.
Figure 3.19 35S promoter specific real time PCR results of positive seed samples
Figure 3.20 35S promoter specific real time PCR results of positive fresh tomato samples
Figure 3.21 NOS terminator specific real time PCR results of positive seed samples
Figure 3.22 NOS terminator specific real time PCR results of positive fresh tomato samples
3.3. Interpretation of the Results

The control of extracted DNA from samples, positive and negative control tomato seeds were performed by patatin gene specific PCR. All DNAs from seed and fresh tomato samples produced the expected 124 bp amplicon indicating the presence and amplification capacity of the samples.

19 out of 35 seed and 25 out of 40 fresh tomato samples did not produce any amplicon with any of the screening and identification primers used in this study. These samples are either not GM or other marker, promoter or terminator regions were used in these samples instead of 35S Promoter, NOS Terminator or NPTII gene. It is known that in literature there are crops improved by using promoter and terminator regions found in plants own wild relatives. Besides, in recent studies marker genes were removed after transformation of the gene.

In screening studies, seed samples 3, 9, 11, 13, 19, 20, 27, 31 and fresh tomato samples 9, 11, 15, 16, 20, 26, 27, 29, 32, 34 produced amplicons with three of the 35S primer sets and seed samples 3, 6, 9, 11, 13, 15, 17, 20, 26, 30 and fresh tomato samples 11, 15, 26, 27 and 34 were amplified with two different NOS specific primer sets. However, seed sample 19 amplified only with nos207 primer set. Furthermore, seed samples 3, 6, 9, 11, 13, 15, 17, 19, 20, 26, 30 and fresh tomato samples 9, 11, 15, 26, 27, 28, 34 and 35 produced amplicons with three different NPTII specific primer sets (Table 3.7 and Table 3.8).

After screening studies, the amplified samples carrying the specific GM construct such as 35S promoter, NOS terminator and NPTII regions were evaluated by means of identification PCRs according to the known genetic map of GM tomatoes. Fresh samples number 15 and 34 and seed samples 11 and 20 could be either containing
PG gene in antisense orientation or containing accd gene or acc gene in sense orientation among other approved ripening delayed tomatoes. However, during identification PCR studies, any amplification signal was not observed for F-type truncated PG and sam-k genes. The amplification of truncated PG gene did not achieved using the primers specific for screening F-type ripening delayed transgenic tomato line. Although these samples were antisense truncated PG gene negative, they might be 1345-1, 8338, Flavr Savr, B or Da type ripening delayed GM tomatoes. During this study, PCR studies were carried out only for screening F-type ripening delayed tomato line. In the future, these samples should be screened for other type of ripening delayed tomato lines such as antisense PG gene inserted Flavr Savr tomato, sense truncated PG gene carrying B type ripening delayed tomatoes, accd gene carrying 8338 and also acc synthase gene inserted 1345-1 transgenic tomato lines.

Besides, seed samples number 15, 26 and 30 could be 35 l N as they carry both NOS terminator and kanamycin resistance gene. However, sam-k specific identification PCR results showed that only positive seed sample SE17 was amplified.

There were a lot of research on Bt crops, however, there were not any detection method for Bt tomatoes in the literature. Cry1Ac gene inserted positive control tomatoes and also the map information were requested from Monsanto, however, they could not provide tomato samples as positive control and any mapping information. Therefore, a primer set was designed for identification of cry1Ac gene which was inserted in 5345-1 GM tomato line by targeting the homologous regions between the natural and synthetic cry1Ac gene sequences and plasmid carrying cry1Ac gene was used as positive control for insect resistant transgenic tomato identification PCR studies. Seed samples 3, 6, 9, 13, 19, 27, 30 and fresh tomato
samples 9, 11, 20, 26, 27, 28, 29, 30, 32, 34 and 35 produced positive signals with designed cry1Ac specific primers. PCR products were sent for sequencing and the results showed high homology (99%) with the expected cry1Ac sequence.

5345-1 line carries PE-35S promoter, NOS terminator and NPTII marker gene but in some of cry1Ac positive samples NOS terminator region was not observed. These samples could be different insect resistant GM line developed by different companies or research institutes by using different terminators. According to the Bruderer et al., (2003), although GM tomato lines with variable improved properties were approved in different parts of the world, there are several approved transgenic tomato lines with no genetic map information.

Although the results obtained both from screening and identification studies show the existence of transgenic tomatoes, several construct specific PCR studies were designed in order to analyze whether these suspected samples were related to the approved transgenic tomato lines or not. Construct specific PCRs were carried out for 351 N and the 5345-1 lines by targeting the junction regions between the gene of interest and the nearest transgenic elements as mentioned before. Amplicons were obtained from both the 351N and 5345-1 targetted construct specific PCR studies. Although expected amplicon was nearly 700 bp for cry1Ac construct specific PCR, the observed band was 550 bp in length. The results were sent to sequence analysis and the results did not show homology with the expected construct sequence. BLAST analysis results for the sequences obtained for the insect resistant GM tomato constructs demonstrated that the primers amplified a segment of tomato genomic DNA. In other words, sequence shows high homology with the sequence of the genomic DNA of the tomato but not with the expected construct sequence. Such kind of result might be obtained because of the unspecificity of the
designed primers, therefore, amplification occurred on the tomato genomic DNA instead of the expected construct region.

Expected amplicon was obtained from the construct specific PCR system designed for 351 N line. Sequence analysis results showed 60% homology with the expected construct sequence. According to the sequence alignment results there were some regions different from the expected sequence. This may be caused from the insertion or deletion of the region in order to modify the construct to get more successful plant transformation event or for high level of gene expression. The PCR products which were sent for sequencing might have some impurities so that some regions might not be sequenced accurately. Therefore in the results obtained from sequencing some regions were not homologous with the expected construct sequence.

In order to obtain more accurate results from the construct specific PCR systems, further studies should be carried out after designing more specific primers for the same and different possible constructs.
<table>
<thead>
<tr>
<th>Seed samples</th>
<th>35S</th>
<th>391bp</th>
<th>207bp</th>
<th>180bp</th>
<th>195bp</th>
<th>479bp</th>
<th>411bp</th>
<th>178bp</th>
<th>35S</th>
<th>NOS</th>
<th>Sam-k</th>
<th>Cry1Ac</th>
<th>pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE1</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE2</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE3</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>SE4</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE5</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE6</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>SE7</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE8</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE9</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>SE10</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE11</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>SE12</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE13</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>SE14</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE15</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>SE16</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>SE17</td>
<td>(-)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>SE18</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>----</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
</tr>
<tr>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>
Table 3.8 Screening and Identification PCR results of Fresh Tomato Samples

<table>
<thead>
<tr>
<th>Fresh samples</th>
<th>Conventional PCR</th>
<th>Real Time</th>
<th>pg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35S</td>
<td>NOS</td>
<td>nptII</td>
</tr>
<tr>
<td>FE1</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>FE2</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE3</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE4</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE5</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE6</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE7</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE8</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE9</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE10</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE11</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE12</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE13</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE14</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE15</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE16</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE17</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE18</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE19</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE20</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
<tr>
<td>FE21</td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
</tr>
</tbody>
</table>
CONCLUSION

Although Turkey is one of the main tomato producers in the world, tomato seeds are imported from several countries. It was known that including the tomato, many genetically modified crop takes place in world food markets and it is really necessary to detect and evaluate the presence of GM foods in Turkish food markets.

In this study, we aimed to screen and identify tomatoes and tomato seeds collected from different Turkish food markets randomly whether they are genetically modified insect resistant tomatoes or not.

As both in tomato and seed samples several transgenic elements are found, identification PCR studies are carried out for 351N and F type ripening delayed GM tomato and also the 5345-1 insect resistant tomato lines. For identification, gene specific and construct specific PCR studies are carried out.

In 18 samples, positive amplification signals were observed indicating the presence of cry1Ac gene. Also, 1 of the samples was produced sam-k specific amplicon. These results were confirmed by sequence analysis.

Although detecting a transgenic element or a gene is not enough in order to say that a sample is GM, as the amplicons could be resulted from any contamination or existence of a false positive result, the possibility of the false positive results or contamination of more than one transgenic elements and specific genes is very low.
However, in order to verify the results for the cry1Ac inserted 5345-1 insect resistant GM tomato line and the sam-k gene inserted ripening delayed 35 1 N line, construct specific studies were carried out. The amplicons indicate the existence of possible constructs. In order to verify the construct sequence, PCR products were sent to sequence analysis. 5345-1 line specific construct specific PCR products sequence analysis results did not show homology with expected sequences. The BLAST analysis of the sequence obtained from the construct specific PCR studies for insect resistant transgenic tomatoes demonstrated that the 550 bp amplicon show high homology with the *Lycopersicon esculentum* genomic DNA. This may caused from the unspecificity of the designed primers that they amplified to the tomato genome rather than the construct. another possibility for this result is that the samples carrying Cry1Ac gene were not related to Monsanto’s 5345-1 line so that the construct was not found in the genome of the samples.

Although the sequence analysis results did not show homology with the expected construct specific to 5345-1 line, cry1Ac gene also more than one transgenic element are found in all suspected samples. The contamination possibility of both 35S promoter, NOS terminator, kanamycin resistance gene and also cry1Ac gene in a sample is very low. As it is maintained before, Turkey imports tomato seeds from Israel, China and USA in high amounts and these countries develop GM crops and the genetic map even the gene constructs of these GM crops are not available. The gene construct of different type of insect resistant transgenic tomatoes developed by different companies or institutes may differ from the 5345-1 line, therefore the sequence results did not match with the expected sequences. In short, suspected samples can be different insect resistant GM tomato line from the 5345-1 line.

Expected amplicon was obtained from the construct specific PCR system designed for 35 1 N line. Sequence analysis results showed 60% homology with the expected
construct sequence. According to the sequence alignment results there were some region different from the expected sequence. This may caused from the insertion or deletion of the region in order to modify the construct to get more successful plant transformation event or high level of gene expression.

In order to obtain more accurate results from the construct specific PCR systems, further studies should be carried out by designing more specific primers for the same and different possible constructs.

Although these obtained data from the screening and identification PCR systems indicates the possible existence of Turkey in GM food market, these results should be validated by an accredited laboratory. Further more specific studies might also be required.
REFERENCES


BATS (Agency for Biosafety Research and assessment of technology impact of swiss priority program biotechnology of swiss national science foundation) “Primer
sequences and amplicon length in PCR-assays to detect GMOs'" (27.08.2007), in http://www.bats.ch/bats/publikationen/1997-2_gmo/tab14_gmo_primer.php


BLAST homology search programme, (27.08.2007), http://www.ncbi.nlm.nih.gov/BLAST/


Canada Food Program- Insect (Lepidopteran species) resistant tomato line 5345, (17.01.2004), http://www.hc-sc.gc.ca/food-aliment/mh-dm/ofb-bba/nfiani/e_2000_tomato.html


ClustalW homology search programme, (28.08.2007), http://www.ebi.ac.uk/clustalw

De Leo Federica, De Leo Francesca, (2005), 9th ICABR International Conference on Agricultural Biotechnology: Ten Years Later, Traceability and Detection of Genetically Modified Organisms in the Labelling of Food Production Chain, EU Directives and Molecular Approaches, Ravello (Italy), July 6-10, 2005


GM Crop database, (10.08.2007), www.agbios.com


Hardegger, M., Brodmann, P., Herrmann, A., (1999) “Quantitative detection of the
35S promoter and the NOS terminator using quantitative competitive PCR” in Eur.

Harris, N., Terry, C.F., (2001) ‘’Event-specific detection of Roundup Ready soya
using two different real time PCR detection chemistries.’’ in European Food
Research and Technology 213, 425–431.

Hemmer, W., (1997) ‘’Foods derived from genetically modified organisms and

PCR systems based on SYBR Green I, Amplifluore and TaqManw technologies for
specific quantitative detection of the transgenic maize event GA21." in Journal of


quantitative event-specific detection of the genetically modified Mon810 MaisGard

for screening and quantification of genetically modified organisms (GMOs)” in
Analytical and Bioanalytical Chemistry 375: 985-993.


Expressing an Insecticidal Protein Derived from Bacillus thuringiensis” in Bio/Technology 11, 194-200


Kumar, H. & Kumar, V., (2004) “Tomato expressing Cry1A(b) insecticidal protein from Bacillus thuringiensis protected against tomato fruit borer, Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae) damage in the laboratory, greenhouse and field” in Crop Protection 23(2): 135-139.


Primer3 primer design programme, (19.06.2007), http://wwwgenome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi


Van der Salm, T., Bosch, D., Honée, G., Feng, L., Munsterman, E., Bakker, P., Stiekema, W., Visser, B., (1994) “Insect resistance of transgenic plants that express modified Bacillus thuringiensis cryIA(b) and cryIC genes: a resistance management strategy” in *Plant Molecular Biology* 26 (1), pp. 51-59


## APPENDICES

### APPENDIX A

### CHEMICALS AND SUPPLIERS

<table>
<thead>
<tr>
<th>CHEMICALS</th>
<th>SUPPLIERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Prona</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>Merck</td>
</tr>
<tr>
<td>Sodium Hydroxide (NaOH)</td>
<td>Merck</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic (EDTA)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrogen chloride (HCl)</td>
<td>Applichem</td>
</tr>
<tr>
<td>Sodium Acetate (NaAc)</td>
<td>Applichem</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone (PVP)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bromphenol Blue</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ethidium Bromide (EtBr)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>Applichem</td>
</tr>
<tr>
<td>Sodium Dodeyl Sulphate (SDS)</td>
<td>Merck</td>
</tr>
<tr>
<td>Ethanol (EtOH)</td>
<td>Merck</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Delta Kimya</td>
</tr>
<tr>
<td>Chlorophorm: Isoamylalcohol</td>
<td>Applichem</td>
</tr>
</tbody>
</table>
CTAB
Tris
ddH2O

Applichem
Sigma
Dr. Zeydanlı
APPENDIX B

BUFFERS AND SOLUTIONS

1. Solutions for DNA isolation

1.1. Hexadecyltrimethyl-Ammonium Bromide (CTAB) Buffer

CTAB 20g/l
NaCl 1.4 M
Tris HCl 100 mM
EDTA 20 mM
Bring the last volume to 1L with dH2O. Adjust pH to 8 and autoclave (121C, 15m.)

1.2. CTAB Precipitation Buffer

CTAB 5g/l
NaCl 0.04 M
Bring the last volume to 1L with dH2O. Adjust pH to 8 and autoclave (121C, 15 m.)

1.3. 1.2 M NaCl

NaCl 70g
dH2O 1 liter
Dissolve in 1000 ml dH2O and autoclave (121C, 15 min).
1.4. Washing Buffer

dH2O 300ml
Pure Ethanol 700ml

2. Buffers for Electrophoresis

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

2M Tris
1M Acetic Acid
100 mM Na2EDTA
48, 44g, 11.8g and 7.45g respectively for 200 ml solution were dissolved in dH2O and pH was adjusted to 8.0. The solution was diluted 50 times before using.

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

0.25 % Bromophenol Blue
0.25 % Xylene cyanol
15 % Ficol 400
40% (w/v) Sucrose
Dissolved in dH2O.

2.3. Ethidium Bromide Solution (Maniatis, 1989)

10 mg/ml EtBr was dissolved in dH2O.
3. Solutions for plasmid isolation

-Solution I
50 mMolar glucose (+4°C), 25mMolar Tris-HCl (pH:8), 10 mMolar EDTA.

-Solution II
0.2 N NaOH and 1% SDS.

-Solution III
3 Molar sodium acetate (pH:8).
### APPENDIX C

#### ENZYMES, MARKERS AND REAGENTS

<table>
<thead>
<tr>
<th>ENZYMES, MARKERS AND REAGENTS</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Taq Polymerase</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>Ribonuclease A (RNase)</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>DNA ladder</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>Deoxynucleotide Triphosphates (dNTPs)</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>XmnI</td>
<td>MBI Fermentas</td>
</tr>
</tbody>
</table>
APPENDIX D

Annealing sites of PatGe-PatGd primer set was illustrated on the Solanum tuberosum gene for patatin with the NCBI accession no. X03932 (Jaccaud., 2003).

2641 gatcaataact attatgtgct gtaagttgcaaa tgcagggcat attaccaat tgcgttgc
2701 cgaaccttgae acaagttgcat caaaaatttc acttcaattaa gtcattgtaat tacaagc
2761 tgggtgttgc ctccattagcc atgcgcacta ctccaggtt tgataaaaaca tataacagc
2821 aagagcagc taatgggggt actgcacagc ggatgtttagt tatacaagaa atgactagttg
2881 cagcaagtctt tacatgaact gatattacc ttctacatgc tttaactgcc ctgatgccac
2941 caaaaaattt cotaaggggt caagtaagct ccaactatatta tttaggggaa ctacataa
3001 ctctcaatag tcgcaatttt acctatatttca caaatttttg atatatcttag gatacatggtt
3061 caaaaattaggt gcgtatagtt cttaatacat ctctattcaag tggattcaaca tggatcctta
3121 gacatcagtc cggattccag atttacattta aagggyltae caagaaaaaa acyagly
3181 cttttattt gatctttggt cottaaggtt agttttggaac cctctcgaac cttgaagctac
3241 ctctaatcctt acctagga ggtcctaaaa caatatatat agataaaaaat tcttattat
3301 gcctttata tacaagggac gtaagtttttt gtttgtgaaace cctttgtcaca cctttaggcc
3361 cgcgcctttgc tagaatatact tagcataacct tttcgctctt ttctcaatttc ggtccacat
3421 ctctcaattgt tcgggtttac cagatactag gatactacaca cagatgctta cagatactagtt
3481 aagttctacag tgcgattttgc atgttatcttg gatacatacag ttactttacaac aectttcaea
3541 ctattttttgt gtttggtgta gcetttatcac atgttatctaa gtaatatattt ttaatatata
3601 gatagccaact tctataaagtt gcggtaagc ctgtaataatt caagaaatata tattatagttg
APPENDIX E

Blue color is indicating the first primer set 35S-F/35S-R that was used in our study, and the pink color is indicating the second primer set, 35S-EUF/35S-EUR and the p35s-af1u7p35s-ar1 primer set is indicated by green color. In some transgenic crops, the duplicated versions of 35S promoter are used (AF078810), the sequence carries a second complementary annealing site for the 35S-1 primer (Jaccaud., 2003).

1  CCTGCAGGTCATGGTGGAGCAGCAGGACACATTGCTCTACTTCTTACACCTTTCTAATATCACAAAGATAC
61  AGTCTAGGAAGACAAAGGCAATTGAGACTTTTCAACAAAGGGTAATATCCCAGACACCT
121  CCTCGGATTCCCATTTGCGGCGCTATCTCTGTCATTATTTGTAAGATAGTGGAAGAGGAAAGG
181  TGGCTCTCTACAAATGCCCATCTGCGATAAAGGAAAGGCACTGTGGAGATGCTCCTCAGC
241  CGACAAGTGCTCCACAAAGATGCAAGGACCCCCACCCACCGAGGGAGCATCCTGTGGAAAGAAAGGACGT
301  TCCAACCACGTCTTCAAAAGCAAGTAGATGGATGTGTATAACATGGGGAGCACGACACACT
361  TGTCTACTCCAAAAATATCAAGGACCAACAGGCTCTCAGAAAGACAAAGGCAATTGAGACTTT
421  TCAACAAAGGTAATATCCCAGAAACCCTCTCGGATTCCATTGGAGAGCCACTTCTGCTACCTT
481  TATTGTGAAAGAATGAGAAGGAGGTGGCTCTCATAAATGCCATCATTTGGGATAAAGG
541  AAAGGCCATCGTTAGAGATGCCTGCGACAGGTGGTCCCAAGATGGACCCCGACCCAC
601 GAGGAGCATCGTGAAAGAGACGTCTTCAACCACGTTGAAAGCAAGGAGTTGATG

661 TGATATCCACTGGTGAGGATGACGCACAATCCCACCTATCTCTTGCAAGACCCCTTC

721 CTCTATATAGGAAGTTTCTTTCATTGAGAGGA
APPENDIX F

Annealing sites of Nos-f/Nos-r (indicating blue) and NOS207F/NOS207R (indicating red) primer sets were illustrated on sequence with the NCBI accession no. U12540.

1 GATCGTTCAACATTTGGCAATAAGTTTTTCTTGAGATTGGAATCTGTTGCGGTCTTGCG

61 ATGATTATCATATAATTTTCTGTTGAATTACGTTAGCATGTAATAATAAACATGTAATGC

121 ATGACGTTATTTATGAGATGGGTTTATGATTAGAGTCCCGCAATTATACATTTAATAC

181 GCGATAGAAAAAATAATAGCGCGCAAAAATAGGATAAAATTATCGCGCGCGGTGTATCT

241 ATGTTACTAGATCG
APPENDIX G

Annealing sites of kanf/kanr (indicating blue), kan411f/kan411r (indicating red) primer sets were illustrated on sequence with the NCBI accession no. AF274974.

1 ATGGATCTGACGAAACCCGTCGAATTGAACAAGATGGATTGGCGACGAGGTTCCTCGGGCCGCT

61 TGGGTGGAGAGGCTATTCCGCTATGACTGGGCAACACGAGAATCGGCTGCTGATGCC

121 GCCGTGTTCCGCTGTCAGCGCAGGGGCCGCGGTTTTTGTCAAGACCGACCTGTCC

181 GGTGCCCTGAATGAACTGCGAGGACGAGGACGCGCGCTATGCTGGCTGCGAGACGGGC

241 GTTCCTTTCGCAGCTGTGCTGACGTGTTGCTCAGTAAGCGGAAGGACTGCGCTTATTTG

301 GCCGAAAGTGGGGGCGGAGATCTCCTGTCTACCTACTGCTTGTGCGGAGGAAATATCC

361 ATCATGGCTGTGCAATGCGGGGCTGATCGCCGCCGCCGCTGATCCGCGGCTACCTGCCCATAACGAC

421 CACCAAGCGAACATCGCATCGAGCGAGACGTACCTCGGATGGAAGCGGGTGCTTGCGAT

481 CAGGATGATCTGCGAAGAGCATCGAGGGGTCTCGCGCCAGCGGAACTGTTTCGCCAGGCTC

541 AAGGCGCCATGCGCCGACGCGAGGATCTCGTGATGACCGGATGCGCTGCTTGCGCG

118
601 AATATCATGGTGAAAATGGCCGCTTTTCGATTCATCGACTGTGGCCGGCTGGGTG

681 GCGGACCGCTATCAGGACATACGTGGCTACCCTGATATTGCTGAAGAGCTCTTGGC

721 GAATGGCCTGACCGCTTCTCCTCGTCTTTACGTTATCGCCGCTCCCGATTCGCAGC

781 GCTTCTATCGCTTTCTTGACGAGTTCTTCTGA

<<<<<<<<<<<

119
APPENDIX II

Annealing sites of sam3f/sam5f primer set was illustrated on sequence with the NCBI accession no. X04791

1 AGCTCTTTGAGTGATCCATATAAACGGAATAATCTCAAGGTGACTGAAAGCCTGGCGCTTA

61 TGATATTTTACCAT?AACGAACTACCATGAGGTAAACACAAATGATTTTCACTAAAGAGCCCTG

121 CGCAGCTCTCTTATTTGTGATTTCCGTTTCCGTCTCTACCTCTTGAGATTGAATA

181 TGAGCAGACACCGCAAACATGTAAGCAGCTTTACGTCGCCCAGCCACCGGTCTTTATGGCTCCGG

241 TTGAGCTACAACGGATTTGAGGCGGTGCTATCGTGAGGCAATCTCAAGCCACACAAACTGAGG

301 AAAAACTGTGTTGGTGTTGCTCGAAAGGCAAAAGGCAAGGCAACTCAATGTTGACCGCCTAG

361 TTTGTAATGAGTGGGACAGATTCTCGTACTGTATACAAATCAGACTCACACGGCCTG

421 GTCTGGTTGTAAGGTTAAAGGTTAGGGCTATCCACGGGAAAGGGCTGACGTCTGCGGGGTAAGTTCC

481 AAGAGTTTCTAAAGGCGACCCGCTGCAAGGCTGCTTCACTATTGATGATGTTGCTGCTG

541 GCAGGAAGTACAAATAAGTGTCTAATCGATAGAGATGCACGAGTGCCTTTATGATT

601 AACATTCCTCTTTATAGAGGTATTGAATG
APPENDIX I

Annealing sites of PG34f/PG34r primer set was illustrated on sequence with the NCBI accession no. X05656.

1 GAATTCAATAGACAAATTTAAAAAAAAACATACCATATACAAATATATCATGTTATCCAAAG

61 GAATAGTATTTCTCCCCATATTATTTTTGCTTTGATCAATCTTTCACTTTCAACCTTTGTTAGAAGCAA

121 TGTTATTGATGACAAATTTATTCAACACAAAGTTTATGATAATATTTCTTGAACAAAGAAATTTTC

181 TCATGATTTTTCAAGCTTATTTTTTCCTTTTAATTGAGCAAAAATATTGGAAAGCAACAAATATAT

241 TGACACAGGTTGATAAAATAGGGAATTAAAGTTGATTAATGTACTATTGCTTTGAGCTAAGGG

301 TGATGGAAAACATATGATAATTGCATTTTGAGCAAGCATGGAATGAAGCATGGTCATC

361 TAGAACAACCTGTCAATTGTGGTTCTCAAAAACAAGAAATATTTCAAGCAAAATCAC

421 CCTTTCCAGGTCATGCAGATCTTTCTATTTCTAGTAAGATTCTTGGAATCTTAAAGAATC

481 TAGTAAAATTTCCAGACTAACAAGATAGAAGGCCTTTGGATTTTGATAGTGTCAAAA

541 TTTAGTGGTTGAGGAGGAGGAACGATATCAATTGCAAATGGCAATGATATGGTGGCCAGTTC

601 TTGCAAATAAAATAAATACATGCATCCAGCAGGGATGCACACCCGGCCTTAAACCCCTTGGAA

661 TTGCAAATAATTGGAAGTGAAATACTTAAAGGATAAAAATGCAAAACAAATTTTACATATCA

721 AATTGAGTCATGCGACTAAATGTTGAGCTTCAAAATTTTGATGATCAATGCTTCAAGCAAGAG

121
781 CCAATACTGATGGAGTCCCACTGTAATCAAATATCTCAATATATTTTATATCTGATACATAT
841 TACTTGAACAGTGGACGATTGAATTCTTTTATTTTGATCTCAAAATGTCAGAGCCAC
901 AAATATTACTTTGCTCAGGATCGTATATATAGTATTGGAAGCTAGGGATCTGGGAATTT Tac
961 AGAAGCTTATGTTGCTAATGCTTTACTGTAATGAAGCCAAATTATCGGTGCGGAAAAATGG
1021 AGTTAGGATAGTCAAGACTTTGGCAGGAGGATCTGGGACAAGCTAGCCACATCTGAAATTTCTGAA
1081 TGTTGGAATGCAAGACTTTACCTTCAATATATATAGGACAAACTATTTTTGATGATCTGATGAGT
1141 TGAAACATGTGATAACAACAGTTTTCACGGGTCAAGTCAAGTAAAAATGTGTTGATGAGAATAT
1201 CAAGGGGCAAAAGTGCAACAAAGGATGGGCCCATAAAAATTTTATGATTGCAGCACAACACTTTCAGG
1261 TAGAAAGAATTAAAATTGGAGAATAAATATATGTTAGGGAAAATGGAACATCACAGAGGC
1321 TACGGGCAAAATGTCCATTTCAACTGATCAGGACATGTTACCCACAGACTGCACCTCATGCT
1381 AGAAATTTTCAGAGGATGAAAGCTCCTTTTTGATATAATTATATTTTATCTATAGATCTTCA
1441 TATATAGCAGATATGATATACAAATAAAAACAAATCTATATCTATGATTGAAATTTAT
1501 TATPTAATATTGATCGGATGTTGGTTAAATAGACTACATGTATTTTTCTATTTTCAGTCA
1561 AAGTTTGACGGATTTGACTTTTTTATAGTCAAAAAATAATAAAATGGTTAT

122
APPENDIX J

Annealing sites of cry323f/cry323r primer set was illustrated on sequence with the NCBI accession no AY525369.

1 ATGGATAACAATCCGAACATCAATGATGCAATTCTTATATAGATTAACCCCTGAA

61 GTAGAAGTATTAGGTGGAGAAGAATAGAATAAATGGTGTACACCCCAATCGATATTTCTTG

121 TCGCTAACGCAATTTCATTTGTGAGTAATTGTCCGGTGCTGGATTTGTGGTAGGACTA

181 GTTGATATAATATGGGGAATTTTTTGGCTCCTCTCAATGGGACGCATTCTTTGTACAATT

241 GAACAGTTAAATACAAAAAGAATAAGAATTCGTAGGAAACAAAGCCATTTCAGATTA

301 GAAGGACTAAGCAACTTGACCAGATCTACGCAGAAATCTTTTAGAGAGTTGGAAGCAGAT

361 CCTACTAATCCAGCATTAAGAGAAGAGATGCTGATTCAATTCAATGACATGAACAGTGCC

421 CTTACAACCGCTATTCCCTCTTTTGTGTCAAATTATCAAGTTTCTCCTTTATCAGTA

481 TATGTTCAAGCTGAAACTTAAACTTATCATGTTCTGAGAGATGGTTCAGTGTTTGGACAA

541 AGGTGGGGATTGATGCCGCGACTATCAATAGTGGTACAAACGACTTGGCCTTATT
1381 GAATTTAATAATATACGATCGGATAGTATTTACTCAAATCCCTGCAGTGAGGGGAAC
1441 TTTCTTTTTAATGGTTCATGTAATTTTCAGGACCAGGATTACTGGGCTGGGACTTAGTTGAG
1501 TTAATAGTAGTGAATGAATAACATTCCAGAATAAGGGTTATATTGAACTTTCAAATTCTC
1561 CCATCGACATCTACCAGATATCGAGTTCGGTGTACGGTATGCTTCTGTAAACCACGGATC
1621 CTCAACGTTAATTGGGTAATTCATCCATTTTTTCCAAATACAGTACCAAGCTAGCTAG
1681 TCATTAGATAATCTACAAATCAAGTGATTTTGGTTATTTTGAAATGGGACTGCAGAGTGATTAA
1741 TCTTCATTAGGTAATATAGTGATGTTAGAAATTITTAGTGGGACTGCAGAGTGATAATA
1801 GACAGATTTGAATTTTTCCAGTTACTGCAACACTCGAGGCTGAATATAATCGTGAAAGA
1861 GGCACAGAGGCGGTGAATGGCGCTGTTACGTCTACAAACCAACTAGGGCTAAAAACAAAT
1921 GTAACGGATTATCATATTGATCAAGTGCTCAATTATGTTACGTATTATTCCGGATGATTTT
1981 TGTCTGGATGAAAAGCGGAGAAATTGTCCGAGAAAAGTCAACATGCGTAA

125
APPENDIX K

Annealing sites of crycsf/crycsr (indicating blue), crycsf/crycsrer (indicating red) primer sets were illustrated on sequence with the NCBI accession no. AY525369 and AF078810.

1  TTCGAGAGTGGGAAGCAACCCGACCAATCTTGAAGAGAGATGCCAGATTCAAT

61  TCAATGACATGAACACGCACGTGACGACCACATTTCGCTTTAGGTGTACC

121  AAGTTCCCTCTTTATCCGTAACGTTACGGCTGCAACTGCGAATTTCTTCAAG

181  ATGTCTCCGGTTCCGAAACACGCTGGGCTGGTGATGCCCAACTATGAATAGTGCCTATA

241  ATGATCTGACTAGGCTATAGTGATACGATGCTGTGTCTGCAGCTACAACACGG

301  GTCTCGAACGTGCTCTTGGGACCCGATTTCTAGAGATTGGGCAAGCACAGTTCCAGGC

361  GAGAGTTGACACTAAACTGTCTTAGACATTGTGTCGCTCTTTGCCAATACGACTCTAGGCC

421  GCTACCCCAATCGGTACTGTGTCACAATTGACCACGGAAATCTACACAAAAACAGTTCTTG

481  AGAACTTCGACGGTAGCTTTGGGACGGCTCAGGCATAAGAGAGACATCAGGTCTC

541  CACACCTGATGGCAATATTGAAACAGTATCACGATCTACACCGATGCCACCCGGCTTTATT

601  ACTACTGTGTCAGGGCATCAGATCATGCGCATCACCCGTTGGGTTCTCTGGAACCAGATTCA

661  CTTTCCCAATCGGGACTATGGCAAGCTCACCACACRGAGTATTGCTGCAAAC
721  TCGTCAGGCGCTGTAATGACCCCTTGCTCCACACTCTATATAGGACACCTTCTCAACATCG
781  GCATCAACAAATCAACAAATTTGCTCTTGCTGCTGACGGAGACAGATTTGGCTATAGGACCTCCT
841  CAAATCTGCACTCCGCTGTCCTACAGAAAAGACGCGGAAACAGGCCATGGATCCTGATGAGATCC
901  CTCCACAGAACAACACGTTCCACCPTAGGCAAGGGTTTAGCCATCGCTTTAAGCCATGTGTTG
961  CCATGTTCCAGGTTCTAGAGTAAATAGCAGCGTTAGTATCATCAGAGCTCCGAGTCTTCT
1021 CTGGGATACATCCTGCTAGTGCTGAGTTTPAAACAACATAATTGCACTCCGATAGCATTACTCAGA
1081 TCCCCAGCTGTAAGGGGAATCTTTTCTTTTAAATGGTGTTCTGTATTTTCAGGACCAGGATTCA
1141 CTGGAGCCGACTTTGATTGGCTGAAGTTCTCTCCGGCAACAAACATCCAGAATAGGAGGTATA
1201 TTGAAGTGCCCATCCTACCTTCATCCACTGCGACATCTACCCAGATATCGTGTTCGTAAGGGTATG
1261 CCTCGGTACCCCTATTGACCTCAAGCGTCAATTGGGTATTTTCCATCTTTTCCAATA
1321 CATGACACGGCAAAGTCAATCTCCCTTTGGATAATATCTCAACATCTAGCGATTTCCGTTACTCCG
1381 AGTCCTACAACTGCTCCCTCTCTGACTGGAAGATAGGTGTGGATAAATTTTCTCCG
1441 GAACCGCGGAGGTGATAATCGACCGCTTCGAAATTCCCTCCCGTACTGCAACGCTCGAGT
1501 AATAGATCTCCTGACGTTCAACATGGTTGGACACGACACACTGGCTATCTCCTCAAAAATAT
1561 CAAAGATAACAGTCTCAGAAGACCAAAGGCAATTAGGACTTTTCAACAAAAAGGTAAATATC
1621 CGGAAACCTCCTCGGATTCCATTCAGCAGCTATCTGTCACTTTATGTGAAGAGTATGTTGGA
1681 AAAGGAAAGGTGGTTCCTTACAAATGCCCATATGTGGATTAAAGGAAAAAGCCTCTGTTGAGA

127
1741 TGCCTCTGCCGACAGTGTTCCCAGAGATGGACCCCCACCACGAGGAGCATCGTGGAAA
1801 AGAAGGACGTTCACCACGCTCTTAAAAATATCAAGATACAGTCTCGAGAGACCACAGGGCAAT
1861 CGACACTTTGTCTACTCAAAAATATCAAGATACAGTCTCGAGAGACCACAGGGCAAT
1921 TGGAGCTTTCAACAAAAAGGTTAATRTCCGGAAACCTCTCTCGGATTTCCATGGGCCAGCTAT
1981 CTGTCAGTTTATTGTGAAGATAGTTGGAAAGGAGGTTGGCTCTTACAAATGGCAGCATTTG
2041 CGATATAAGGGAAAGACCATCGTTGAAGATGCTCTCCTGCGGCAAGTGTTCCCAGAGATGGACC
2101 CCCACCCAGGAGCAGCTCTGAGAAAGAGGAGAGCTTCCACCCAGGCCTTTCAAGAAGGCT
2161 GGATGGATGTGATATCTCCACTGAGTAAAGGAGGACACCAACAATCCGACTATTTCTGCCA
2221 AGAACCCTTCTCTATATAGGAAGTTCATTTCATTGAGGACGA

1 CCACCTAGGCAAGGATTTAGTCATCGATTACGCAATGTGTTTCCAGGCTTT
61 AGTAATAGTATGTAAGATATAAAAAGAGCTCTCTATGTCTCCTTTGATACATCGTACTGCT
121 GAATTTAAATAATAATATCGCATGGATAGTATTACATGAAATCCGAGTCAGTGAGGAAAC
181 TTTCTTTTTATGTTTACGATTAAATTTCCAGGACCAGGATTTAGTTGGGGGACTTAGTTAG
241 TTAATAGTAGTGGAAATAAACATTGAGAAAPAGAGGATATTATGGAAGTTTCCAATTCATCT
301 CCATCGACATCTCCAGATATCGGATTGTTAGGATGCTCTTGTATGACCTTACGCCAGTAC
361 CTCAACGTTAATTGGGTATTGATCCATTTTTTCCAATGATACACGCTAGTACACCTAC
421 TCAATTGATAATCCAAATCAAGTGTTTTGATTATTTGAAAAAGTGCCAAATGCTTTTACA

128
481 TCTTCATTAGGTAAATATAGTAGGTTAGAGAAATTTAGTGGGACTGCAGGAGTGATAATA
541 GACAGATTTGAATTATTTTACGTTACTGCAACACTCGAGGGTGTAATATATCGTGAAAGA
601 GCCGAGAAAGCGGGTGAATGGCGCTTACGCTCACAACCCAAACTAGGGCTAAAACAAAAT
661 GTAACGGATTATATCATATTGTACAGTTTATGCCAATTAGTATTACGGTAGAATTTT
721 TGGCTGGAAGAAAGCGGAAATTTGTCGAGAAAGTCAAAACATGCTAACCCAGGTCA
841 CCAAAAGGGCATTGAGACTTTTTCACACAAAAGTTAACATCGGAAAACCTCTCCAGATTCA
901 TTGGCCAGCTATCGCTACTTTTTATTGGAAGTAGAGTTGGAAAGGAAGGTTGGCTCCTCAAA
961 ATGCCATCTCGGATAAAGGAAAGGCCATGGAGATGGCCTGCGGAGAGCTGGTCCC
1021 CAAAGATGGACCCCAACCCACCCAGAGGACATCGTGAGAAAAGAAGACGTTCACAACCACGT
1081 TTCAAGACGAGTTGATGTGATTACATGGTTGGAGCAGCACACACTCATTGCTACTCCAA
1141 AAATAATAAGGATAACGTCGAGGAAAGCAAGGCAATTGAAGACCTTTTCAACAAAGGTT
1201 AATACCCGGAAACCTCCTCCGATTCCTCAGCCACTATCTGTACTTTTTATTGGAAGAT
1261 AGTGGAAAAGGAAGGTTGGCTCTACAAATGCCCATATTGCGATAAAGGAAGGCGATCGT
1321 TGAAAGATGCCCTCTGCCGACATGTTGCCAAAGATGGACCCCCACCCACAGGAGGACATCGT
1381 GGAAAAGGAAGACGTTCACGCACAGTGATTGGATGATGATATCTCCAC
1441 TGACGTAAGGGAGTAGGCACAAATCCACTATCTGCCAGAACCCCTTCTCTATATAAGG
1501 AAGTTCTTTCTTTGGAGAGGA
APPENDIX L

Arrows are indicating the primer set sameul/sameur by targetting the sequence with
the NCBI accession no. U12540 and X04791.

```
1  AGCTCTTTGAGTGTAACCATATAAGCGAATACAACGACTGAAAGCGTGCCCTTTA
61  TAGATAATCACTTAACCGAACACTGAGACTAACCCAGATATGATTTTCACAAGAGCTG
121 CGCACGTTTTATGACTGTTTCCCGTTGTTGGCATGGTGAATA >>>>>>>>>>
181 TGAAGCAGACACCGCCACATGGTAAAGCATTGAGATGCGACCGGCTTTATGCGTCCG >>>>
241 TTGACTGAACCGAATTGGACCGCTCATTGCGAGCAATTCTGACACCGACTGAGG
301 AAAAAACTTGTTGCTGACGCTGCAAGCAAAAGCGCGACGGGACTCAATGTTGCAGCGCTAG
361 CTGTGAAATGAGGGGACCAAGGTTGCGCTGACTGATCCAAAATCGGCTGCAGGCCTG
421 GTCTGGTGTACGCTAAAGGTATCGACGGTTAAGCGTGAAGCGCTGGGCTAGTTCC
481 AAGGGTTTCTAAAGGGCGAGCTGCAAGGCTGCTTACTATGATGAAGTCCGCTCC
541 CCTGCCAACACTACAATAACTGTTAAAACACTCAAGGCTCAAGCGATGCCTGCGCTTTATGATT
601 AAGAAAATCTCTCTCTAGAGATTTGGAATGGACCTGTTCAAGAACATTTGGCATAAAGTTCTT
661 AAGATTTGATCCGCCGCTGTCCCGTGTTCCGATTACATTATATATATATTTTCTGGAAGTTACGT
721 TAAGCATGTAATTAAACATGTAATGCGAAGTTTTTTATGACAGCAGGTGTTTTATGAT
781 TAGAGTCCGCCAATTATACATTTAATTACGCGATAGAAAAACAAATATAGCCCGCAAAC
<<<<<<<<<><<<<<<<<<<><<<<<<<<<<
841 GGATGAAATTATCGCGCCCGCTGTCCATCTATGTTACTAGATCG
```
APPENDIX M

Sequence alignments of natural vs. synthetic cry1Ac genes. DQ285666 and EF094884 indicate natural cry1Ac and AY525369, U63372 synthetic cry1Ac gene sequences.

```
clustalw.out

CLUSTAL W (1.83) multiple sequence alignment

DQ285666.1  AFAAXGCGCCGAGATAGTAAATGTTACACCCCTGGCTCCAAATAGATATGTTAA
EF094884     ..............................................................
AY525369     ..............................................................
U63372       ..............................................................

DQ285666.1  AATTCTTGACTCTGATCTTCTAATATTCTAAATGTTACACCCCTGGCTCCAAATAGATATGTTAA
EF094884     ..............................................................
AY525369     ..............................................................
U63372       ..............................................................

DQ285666.1  AAGAAAAGCCTTAGCTTCTTCTAATATTCTAAATGTTACACCCCTGGCTCCAAATAGATATGTTAA
EF094884     ..............................................................
AY525369     ..............................................................
U63372       ..............................................................

DQ285666.1  AAGAAAAGCCTTAGCTTCTTCTAATATTCTAAATGTTACACCCCTGGCTCCAAATAGATATGTTAA
EF094884     ..............................................................
AY525369     ..............................................................
U63372       ..............................................................

DQ285666.1  AAGAAAAGCCTTAGCTTCTTCTAATATTCTAAATGTTACACCCCTGGCTCCAAATAGATATGTTAA
EF094884     ..............................................................
AY525369     ..............................................................
U63372       ..............................................................
```

132
APPENDIX N

Figure A.1 35S Promoter specific PCR results using 35Sf/r primer set: line1: 100 bp ladder, line2: PSG, line3: FE15, line4: SE9, line5: SE1, line6: No template, line7: 100 bp ladder.

Figure A.2 35S Promoter specific PCR results using P-35S-af1u/p35s-ar1 primer set: line1: 100 bp ladder, line2: no template, line3: SE1, line4: SE9, line5: SE11, line6: SE13, line7: SE19, line8: SE27, line9: SE31, line10: PSG, line11: SE20, line12: FE9, line13: FE9, line14: FE11, line15: FE20, line16: FE26, line17:

Figure A.4 NOS Terminator specific PCR results using NOSf/r primer set: line1: 100 bp ladder, line2: PSG, line3: SE15, line4: SE4, line5: SE5, line6: SE13, line7: FE34, line8: SE17, line9: FE27, line10: SE20, line11: FE27, line12: FE26, line13: 100 bp ladder.
Figure A.5 NOS Terminator specific PCR results using NOS207f/r primer set:
line1: 100 bp ladder, line2: SE1, line3: SE2, line4: SE14, line5: SE10, line6: SE16,
line7: SE7, line8: SE26, line9: SE30, line10: FE12, line11: FE25, line12: FE29,
line13: SE18, line14: FE13, line15: FE32, line16: SE7, line17: SE10, line18: SE28,

Figure A.6 NOS Terminator specific PCR results using NOS207f/r primer set:
line1: 100 bp ladder, line2: SE3, line3: SE10, line4: SE14, line5: SE16, line6: SE21,
line7: SE22, line8: SE20, line9: SE23, line10: SE24, line11: FE8, line12: FE6,
line13: FE14, line14: FE17, line15: FE20, line16: FE22, line17: SE27, line18: SE32,
line19: SE1, line20: SE33, line21: No template, line22: 100 bp ladder.

Figure A.8 NptII specific PCR results using KANf/r primer set: line1: 100 bp ladder, line2: FE15, line3: PSG, line4: FE12, line5: FE1, line6: SE1, line7: 100 bp ladder.
Figure A.9 NptII specific PCR results using KAN411f/r primer set: line 1: 100 bp ladder, line 2: SE3, line 3: PSG, line 4: FE15, line 5: SE5, line 6: SE6, line 7: FE26, line 8: SE12, line 9: SE17, line 10: SE7, line 11: PSG, line 12: SE20, line 13: SE26, line 14: FE9, line 15: FE27, line 16: FE34, line 17: 100 bp ladder.